Vol. XLIII No. 10

October 1954

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

CONTENTS OF THIS ISSUE

The Determination of Inositol as the Hexaacetate 577
F. J. Bandelin, H. E. Deane, and *R. E. Pankratz*

A New Partition Chromatographic Procedure for the Assay of Pharmaceuticals 580
Daniel Banes

Sabadilla Alkaloids. IV. Separation of Veratridine and Cevadine by Partition Chromatography 584
Glenn R. Svoboda and *Lloyd M. Parks*

Analysis of Mixtures of Theobromine and Caffeine by Spectrophotometric Method 589
James W. Miles and *Duane T. Englis*

Blood Sugar Levels and Urinary Excretion of Intravenously-Infused Glucose, Fructose, Galactose, and Sorbitol 592
Victor P. Seeberg, Barbara Whitney, and *Dorothy Goldman*

The Physics of Tablet Compression. IV. Relationship of Ejection, and Upper and Lower Punch Forces during Compressional Process: Application of Measurements to Comparison of Tablet Lubricants 596
Eino Nelson, S. M. Naqvi, L. W. Busse, and *Takeru Higuchi*

Amides of 5-Nitro-2-Thiophenecarboxylic Acid 602
William O. Foye and *John J. Hefferren*

Ultraviolet Irradiation and Absorptiometric Methods for the Determination of Diethylstilbestrol in Tablets 605
John M. Goodyear, L. Shirl Hatfield, and *Max M. Marsh*

The Coloring of Mint Spirits 609
Robert J. Donovan, Leslie M. Ohmart, and *Mitchell J. Stoklosa*

The Genetic Basis of the Ketones, Carvone, and Menthone in *Mentha Crispa* L. 612
Merritt J. Murray and *Robert H. Reitsema*

Chemical Examination of *Podophyllum emodi* Wall. var. *hexandrum* (Royle) 614
S. C. Chakravarti and *D. P. Chakraborty*

The Comparative Anticholinergic Activity of a Series of Derivatives of 3-Hydroxy Piperidine 616
John P. Long and *Hugh H. Keasling*

Substituted Xanthines. I. Preparation and Properties of Some Choline Theophyllinates 619
B. F. Duesel, H. Berman, and *R. J. Schachter*

In vitro Antimicrobial Activity of a Dehydroacetic Acid and *p*-Chlor-*m*-xylenol Combination 622
R. L. Stedman, S. L. Engel, and *I. M. Bilse*

Piperazines. IV. 1-Heterocyclic-4-substituted carbamyl-and-thiocarbamylpiperazines 625
E. A. Conroy, N. Q. Quinones, K. L. Howard, H. W. Stewart, and *J. J. Denton*

Piperazines. V. 1-Heterocyclic-4-monoarylcarbamyl- and -monoarylthiocarbamylpiperazines 628
K. L. Howard, E. A. Conroy, H. W. Stewart, and *J. J. Denton*

Isotonic Solutions. III. Amino Acids and Sugars 632
Thaddeus S. Grosicki and *William J. Husa*

Gallic Acid Ethanolamide (N-(β-Hydroxyethyl)-3,4,5-trihydroxybenzoic Acid Amide) 635
Gerhard Julius Haas and *Margaret Rose Zentner*

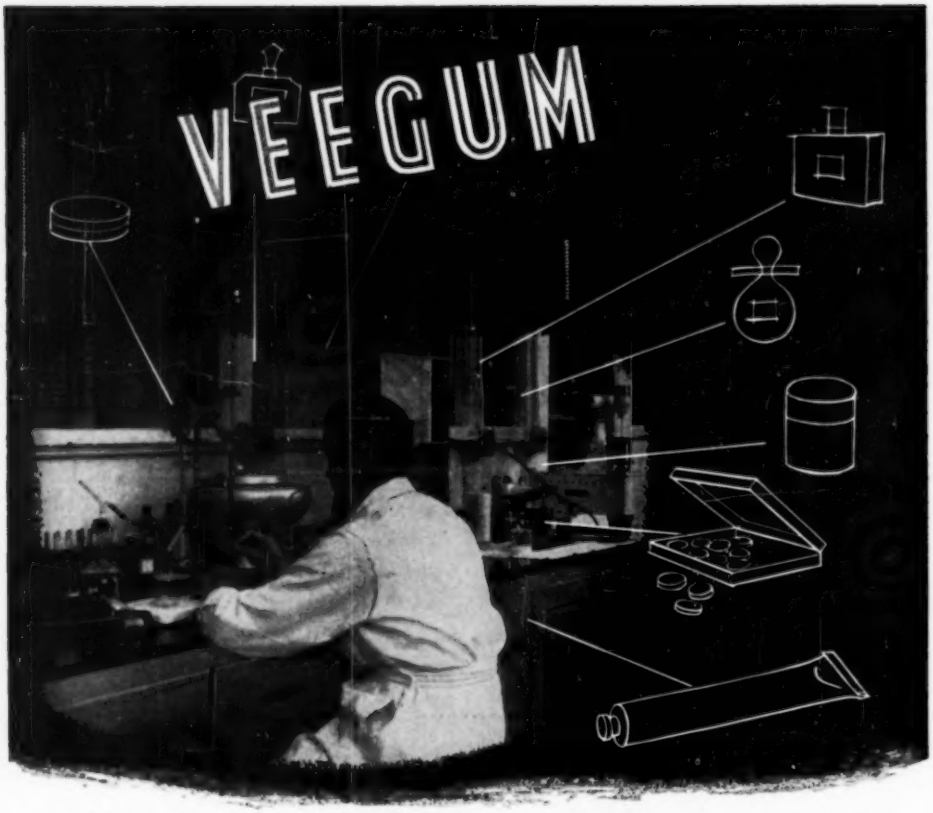
A Note on the Effects of Feeding Saw Palmetto Berries, Serenoa Repens (Bartram) Small, to Rats 636
Seldon Dick Feurt and *Lauretta E. Fox*

Book Notices 638

Advertising ..... Cover pages 2, 3, 4 and I, IV, V, VII and VIII

New and Nonofficial Remedies ..... II, III and VII

Copyright 1954, by the American Pharmaceutical Association



A Versatile Physical Conditioner For All Your Pharmaceuticals

VEEGUM enhances the physical qualities of your tablets, powders, pastes and liquids. Inorganic, non-toxic, non-irritating, VEEGUM is a completely stable suspending and emulsifying agent. Highest quality and uniformity is assured by the rigid control of the R. T. Vanderbilt Company laboratories.

IN YOUR OWN LABORATORY, you will find VEEGUM the most efficient agent for:

BINDING AND DISINTEGRATING TABLETS: An aqueous dispersion of VEEGUM, sprayed in at the granulating stage, will bind most tablets without extra operations. Its disintegrating action requires only one tenth as much VEEGUM as starch or other disintegraters.

LUBRICATING MEDICINAL POWDERS: Add the VEEGUM as a dry powder; very little is needed.

SUSPENDING HYDROPHOBIC SOLIDS: VEEGUM suspends at lower viscosity than organic gums. Thixotropic characteristics give added suspending ability.

EMULSIFYING LOTIONS and OINTMENTS: Dispersions of VEEGUM are effective emulsifiers with or without heat. Small amounts of VEEGUM stabilize many types of emulsions over a wide range of pH.

MAINTAINING PRODUCT CONSISTENCY: VEEGUM has the unique property of thickening with heat to maintain product consistency at higher than normal temperatures.

SEND TODAY FOR THE VEEGUM STORY.

R. T. VANDERBILT CO.
SPECIALTIES DEPARTMENT
230 PARK AVENUE **NEW YORK 17, N. Y.**

☐ Please send VEEGUM Bulletin B53. ☐ Please send sample of VEEGUM.
☐ Please send information on using VEEGUM for:_____
_____
(state application)

NAME_____

POSITION_____
(Please attach to or write on your company letterhead)

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

SCIENTIFIC EDITION

Committee on Publications

Don E. Francke, *Chairman*
Walter M. Chase
Robert P. Fischelis
F. Royce Franzoni
Hugo H. Schaefer

Editorial Advisory Board

John E. Christian
Walter C. Gakenheimer
George P. Hager
Ewart A. Swinyard
Elmer B. Vliet
H. W. Youngken, Jr.

JUSTIN L. POWERS, EDITOR

ANNOUNCEMENT OF OPEN CONFERENCE ON N. F. X

An open conference on drug monographs to appear in the tenth edition of the National Formulary will be held at the Sheraton Park Hotel, Washington, D. C. beginning at 2:00 p. m. on Wednesday, November 10, 1954. This conference is designed to enable all interested persons to offer comments. The conference will be restricted in scope to the N. F. X drug monographs. Other parts of the N. F., identical with those of the U. S. P., will be covered in a U. S. P. Open Conference on November 11 and 12, also to be held at the Sheraton Park Hotel.

The Profession, Science and Business of Pharmacy

offers exceptional opportunities to your apprentice, son or daughter, for a successful career following B.Sc., M.Sc., D.Sc. degree courses at this institution.

Tomorrow

Philadelphia

oldest yet most modern of its kind in the Americas. Schools of Chemistry, Bacteriology and Biology also. *Write for free catalog.*

COLLEGE OF PHARMACY AND SCIENCE

43rd St., Woodland and Kingsessing Aves., Philadelphia 4, Penna. Founded in 1821.

The Journal of the American Pharmaceutical Association is published monthly by the American Pharmaceutical Association in two editions: Scientific Edition and Practical Pharmacy Edition, under the supervision of the Committee on Publications of the Council of the Association. Publication office: 20th and Northampton Streets, Easton, Pennsylvania.

Editorial and Executive Offices—2215 Constitution Avenue, N. W., Washington 7, D. C. Robert P. Fischelis, Secretary and General Manager.

Annual Subscription—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition and Scientific Edition combined: United States, Canada, Pan America, Spain, and Philippines, $10.00; other foreign, $12.00.

Journal of the American Pharmaceutical Association, Scientific Edition alone: United States, Canada, Pan America, Spain, and Philippines, $6.00; other foreign, $7.00.

Journal of the American Pharmaceutical Association, Practical Pharmacy Edition alone: United States, Canada, Pan America, Spain, and Philippines, $5.00; other foreign, $6.00.

Members of the American Pharmaceutical Association receive the combined subscription as a part of their annual dues. Members may request omission of either edition, but this will not reduce the amount of the annual dues.

Single Copies—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition: United States, Canada, Pan America, Spain, and Philippines, $0.50; other foreign, $0.60. Scientific Edition: United States, Canada, Pan America, Spain, and Philippines, $0.60; other foreign, $0.70.

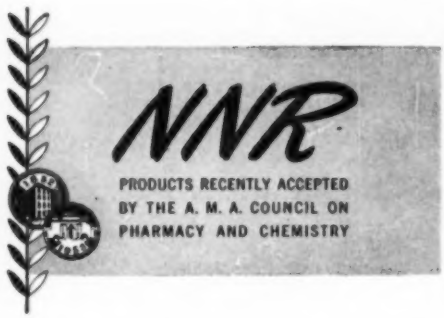
Back issues of unbound Journals same price as current volume.

Subscription rates are subject to change without notice.

Change of Address—Members and subscribers should bear in mind that second class matter is not forwarded by the Post Office and should supply both the Post Office and the American Pharmaceutical Association with any change of address immediately upon its occurrence.

Claims for missing numbers will not be allowed if received more than 60 days after the date of issue, or if loss was due to failure to give 30 days' notice of change of address. Claims asserting that Journals are "missing from files" cannot be allowed. The Association cannot accept responsibility for foreign delivery when its records indicate that shipment has been made.

Entered as second class matter January 23, 1917, at the Post Office at Easton, Pennsylvania, under the Act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the 5th. Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.



CARBOMYCIN.—Magnamycin (Pfizer).—Carbomycin is an antibiotic isolated from the elaboration products of *Streptomyces halstedii*, when grown by deep culture in suitable media. The structural formula of carbomycin has not been established.

Actions and Uses.—Carbomycin possesses strong inhibitory activity against certain Gram-positive bacteria. Its activity against other types of bacteria and other microorganisms is under investigation. Until adequate clinical evidence becomes available, carbomycin is indicated only in the treatment of infections caused by staphylococci, pneumococci, and hemolytic streptococci. Therefore, it is useful in the treatment of pneumonia, urinary tract infections, soft tissue infections, abscesses, and tonsillitis caused by these organisms. Its usefulness in bacteremia and septicemia has not been completely evaluated, but it can be employed in these conditions also when the causative organisms are found to be susceptible on the basis of sensitivity tests.

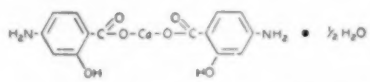
Carbomycin, as the free base, is only slightly soluble in water but is readily absorbed following oral administration. Blood levels produced following oral administration are not significantly lower than when water-soluble acid salts of the drug are administered by intramuscular injection. An appreciable amount (approximately 10% of the ingested dose) is excreted in the urine in active form, and the drug appears to be distributed throughout the body in all organs and secretions. Following intravenous administration of its soluble salts, the drug disappears rapidly from the blood stream. The ultimate disposition in the body of the unexcreted portion has not been determined.

Carbomycin exhibits a low degree of toxicity in experimental animals. Clinically, no harmful side effects have been observed with therapeutically effective doses. Studies of the urine, blood, and liver function have revealed no evidence of toxic action. Nausea and vomiting are the principal side effects; diarrhea occurs infrequently. As for all new drugs, close clinical observation for undiscovered toxic effects is desirable; and, for periods of therapy extending beyond two weeks, repeated blood counts should be performed. As with other antibiotics, its use may result in overgrowth of nonsusceptible organisms, particularly *Monilia*. If new infections caused by nonsusceptible bacteria or fungi appear during therapy, the drug should be discontinued and/or appropriate measures instituted.

Dosage.—Carbomycin is administered orally; optimal dosage is still under investigation. For adults the present total daily dosage is 2 Gm. divided into four equal doses given every six hours; in urinary tract infections and in some soft tissue infections, 1 Gm. daily may be adequate. When infections do not respond to 0.5 Gm. every six hours, the dosage may be increased to 1 Gm. every six hours. Duration of therapy is governed by the clinical response and should be continued until temperature, pulse, and respiration have been normal for forty-eight hours and until other acute manifestations have subsided. The dosage for children is also under study; carbomycin is presently given on the basis of 50 to 100 mg. per kilogram of body weight daily, divided into four equal doses administered at six-hour intervals.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **155**, 1335 (August 7, 1954).]

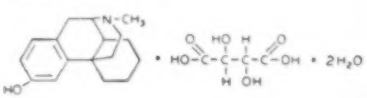
CALCIUM *p*-AMINOSALICYLATE.—The structural formula for calcium *p*-aminosalicylate may be represented as follows:



Actions and Uses.—Calcium *p*-aminosalicylate shares the actions and uses of *p*-aminosalicylic acid and sodium *p*-aminosalicylate. See New and Nonofficial Remedies under the monograph on *p*-aminosalicylic acid. Calcium *p*-aminosalicylate has no established advantage over the sodium salt, except that it can be administered to patients on a sodium restricted diet. Its therapeutic activity is considered equal to that of sodium *p*-aminosalicylate. The incidence of gastric intolerance or other side-effects has not been shown conclusively to be less with the calcium than with the sodium salt.

Dosage.—Calcium *p*-aminosalicylate should be administered orally, and the dosage should not be less than that usually recommended for the acid or for the sodium salt: 12 to 15 Gm. daily, divided into not less than three equal doses of 4 to 5 Gm. each, given every eight hours, before or preferably between meals. Theoretically, the dose of the hydrated calcium salt should be about 25% larger than the usual dose of the acid to provide an equivalent amount of the drug, whereas the hydrated sodium salt would require only a 15% larger dose than the acid. However, a total daily dose of 15 Gm. of the sodium *p*-aminosalicylate yields about 1.6 Gm. of sodium that makes the sodium salt unsuitable for *p*-aminosalicylic acid therapy in patients who are required to restrict sodium intake.

LEVORPHANOL TARTRATE.—Levo-Dromoran Tartrate (Hoffmann-LaRoche).—$C_{17}H_{23}NO \cdot C_4H_6O_6 \cdot 2H_2O$.—M.W. 443.48—*l*-3-Hydroxy-N-methylmorphinan tartrate dihydrate.—The structural formula of levorphanol tartrate may be represented as follows:



Actions and Uses.—Levorphanol tartrate, a potent, synthetic analgesic related chemically and pharmacologically to morphine, produces a similar

intensity of analgesia in much smaller doses and seems to be somewhat longer-acting. Available experimental evidence indicates that the toxicity of levorphanol roughly parallels its analgesic activity. With corresponding analgesic doses, its margin of safety is approximately equal to that of morphine.

Levorphanol tartrate is useful for the relief of severe pain and may be employed for the management of intractable pain caused by cancer and other tumors, severe trauma, biliary and renal colic, gangrene, and myocardial infarction. It is also useful for preoperative medication and postoperative relief of pain.

Levorphanol tartrate produces side-effects similar to those of morphine, except that it is less likely to cause constipation. Pruritis or sweating occurs infrequently. Nausea, emesias, and dizziness occur more commonly in ambulatory patients, as occurs with the use of other narcotic analgesics. The contraindications are the same as for morphine. Because the drug exhibits an addiction liability similar to that of morphine, the same precautions should be observed as for other addicting analgesics.

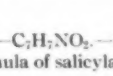
Dosage.—Levorphanol tartrate is administered either orally or subcutaneously. The recommended average adult dose is 2 to 3 mg. Dosage may be subject to adjustment, in accordance with the age and weight of the patient, the severity of pain, and the development of tolerance. As with other addicting analgesics, initial dosage should be as low as possible in the management of intractable pain to delay the development of tolerance.

Tests and Standards.—

Physical Properties: Levorphanol tartrate (hydrated) is a white, odorless, bitter, crystalline powder, m. p. 114–116°. It is very slightly soluble in chloroform and ether. The approximate amounts that dissolve at 25° in the following solvents to form 100 ml. of solution are 0.9 Gm. in alcohol and 2 Gm. in ether. Levorphanol tartrate is stable to light, air, heat, and moisture. The *p*H of a 0.2% solution is 3.4–4.0.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **156,** 250(September 18, 1954).]

SALICYLAMIDE.—$C_7H_7NO_2$.—M.W. 137.13.—The structural formula of salicylamide may be represented as follows:



Actions and Uses.—Salicylamide, the amide of salicylic acid, shares the actions and uses of acetylsalicylic acid (aspirin). Its analgesic potency is no greater and may be somewhat less than that of aspirin. Its antipyretic and anti-inflammatory or antiarthritic properties are not superior to those of aspirin. The over-all incidence of gastric intolerance to salicylamide is about the same as, or a little less than, that to aspirin; however, patients allergic to aspirin have been reported not to be sensitive to salicylamide. It can be used safely in place of salicylates whenever such medication is indicated.

Salicylamide is readily absorbed from the gastrointestinal tract, but does not produce high salicylate levels in the serum. It is diffused widely throughout the tissues, excreted chiefly by the kidneys, and apparently is not destroyed appreciably in the body. Since the toxicity of salicylamide compares closely with other salicylates, it should be employed with the same general precautions. The possibility of the development of sensitivity to salicylamide after its repeated use, particularly in patients already allergic to other salicylic acid compounds, should be kept in mind.

Dosage.—Salicylamide is administered orally, preferably after meals and with fluids to minimize gastric irritation. The dosage should not be less than that for aspirin. As a simple analgesic or antipyretic, single doses of 0.3 to 1 Gm. three times daily may be adequate; as an antirheumatic agent, doses of 2 to 4 Gm. three times daily (or 1 to 2 Gm. every four hours) may be prescribed, according to gastric tolerance, over periods of three to six days. For children, correspondingly smaller doses should be employed.

Tests and Standards.—

Physical Properties: Salicylamide is a white, almost odorless, crystalline solid, m. p. 139–142°. It is freely soluble in alkalis. The approximate amounts that dissolve at 25° in the following solvents to form 100 ml. of solutions are 7 Gm. in alcohol, 1 Gm. in chloroform, 3 Gm. in ether, 5 Gm. in propylene glycol, and 0.2 Gm. in water. Salicylamide is fairly stable to heat, moisture, and light.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **156,** 42(September 4, 1954).]

TRICHLOROETHYLENE—U. S. P.—The structural formula of trichloroethylene may be represented as follows:

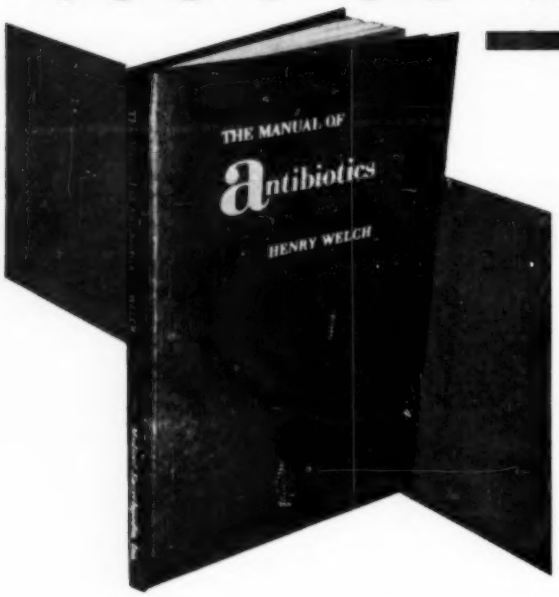
Actions and Uses.—Trichloroethylene is a volatile liquid that produces prompt analgesia and anesthesia when inhaled. Its action resembles that of chloroform but is more rapid and less potent. It is suitable for inhalation as an analgesic agent only. Anesthetic concentrations do not produce complete muscular relaxation and are associated with tachypnea and bradycardia, sometimes accompanied by extrasystoles. These are signs of overdosage. Tachypnea is a sign that the first plane of anesthesia has been reached and should be regarded as a warning that administration has exceeded the analgesic level. With a suitable inhaler, self-administration under professional supervision is considered relatively safe for producing analgesia, usually without unconsciousness. Patients may experience slight dizziness and numbness the first few minutes. Irritation of respiratory passages (excessive salivation or secretion of mucus), nausea, and vomiting are infrequent. If sufficient vapor is inhaled to produce unconsciousness, the mask automatically falls away from the face to prevent overdosage. Consciousness usually is restored within twenty to thirty seconds. Self-administration should not be permitted while the patient is alone.

Trichloroethylene as an analgesic mixture with air or oxygen is useful in obstetrics during labor and for delivery in conjunction with pudendal block or low spinal anesthesia. It also may be employed as an analgesic agent in minor surgical and dental procedures and in major operative procedures as an adjunct to light general anesthesia produced by other agents. When this drug is administered with

(Continued on page VII)

Just Off the Press...

1954-55 MANUAL



For the first time, there are gathered between the covers of one book a list of the preparations, therapeutic index, generic and trade names, and names and addresses of producers of all existing antibiotics and their preparations.

This information, prepared under the editorial direction of Henry Welch, Ph.D., and published by the Medical Encyclopedia, Inc., is distributed exclusively by the American Pharmaceutical Association.

Lists Ingredients, Trade Names, Indications, and Producers

Practicing pharmacists will welcome this authoritative list of antibiotics and their products, complete as of the date of publication. It provides the list of antibiotics in alphabetical order with the active ingredients, the trade names, the indications and the names of the producers.

For ready reference there is an index of trade names in this field, an index of generic terms applied to antibiotics, and an index of the manufacturers of these products, giving the name of the firm and the address.

To identify a trade name it is only necessary to check the trade name index. Opposite the trade name in question is the page number on which the generic term is given, along with the active ingredients and indications for the preparation. All other trade names that have been assigned to each preparation are also shown.

OF ANTIBIOTICS . .

Multiplicity of Trade Names

In the preface to this manual, Dr. Henry Welch, the author, states: "Over the past few years the number of trade names used for antibiotic preparations has increased tremendously. For example, one single penicillin preparation has as many as thirty-six trade names. In fact, the number of trade names for the same drug is limited only by the number of manufacturers. Because of this multiplicity of trade names it has become practically impossible for the physician, pharmacist, and others engaged in the use of these drugs to remember from the trade name just what the composition of the product is."

The American Pharmaceutical Association is pleased to make this manual available for general distribution as a service to the pharmaceutical and medical professions and to the drug industry.

$2.50—Less 20% Discount to A.Ph.A. Members

The price of the manual is $2.50. A discount of 20 per cent is allowed members of the American Pharmaceutical Association in good standing.

All orders for this publication should be addressed directly to the American Pharmaceutical Association, 2215 Constitution Avenue, N.W., Washington 7, D. C., **and should be accompanied by check or money order to cover the cost, which includes mailing.**

MAIL COUPON Now!

American Pharmaceutical Association S
2215 Constitution Ave., N. W., Washington 7, D. C.

Gentlemen:

I enclose check (or money order) for $2.50 ($2.00 if now a member in good standing of the A. Ph. A.) for which please send me POSTPAID, a copy of the 1954-55 Manual of Antibiotics.

Name.....

Address.....

.....

1955 Iodine Research Award Nominations Requested Prior to January 1, 1955

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1955 Chilean Iodine Educational Bureau, Inc. Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1955 Award, *nominations must be received on or before January 1, 1955.*

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council. The present committee includes Justin L. Powers, chairman; Paul Block, E. A. Brecht, Leroy D. Edwards, Harvey B. Haag, Lloyd M. Parks, and Heber W. Youngken.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its seventh year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine in Brooklyn received the sixth award which was conferred during the 1954 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

N.N.R.

•••••••••••••••••••from page III

inhalation anesthetics, standard anesthetic machines with a closed circuit may be used, provided they are adjusted so that trichloroethylene does not come into contact with soda lime. If contact with soda lime occurs in closed apparatus, persistent trigeminal neuralgia and other cranial palsies may result from the formation of toxic degradation products (acetylene dichloride, phosgene, and carbon monoxide), formed by the interaction with soda lime.

Until further experience is gained, trichloroethylene is not recommended for use in patients with severe cardiac failure, active cardiac disease, or toxemia of pregnancy. It should never be employed for induction anesthesia. Administration of epinephrine should be avoided whenever trichloroethylene is used.

Dosage.—Trichloroethylene is administered by inhalation by means of an inhaler device controlled by the patient or a mask or closed-circuit anesthetic machine controlled by an anesthetist. Premedication can be carried out according to the preference of the physician. During labor or for minor surgical procedures, 10 to 12 self-administered inhalations from a suitable device are taken by the patient at the onset of pain. When this agent is dropped on a mask or placed in a machine, a minimal concentration should be maintained at all times to avoid even the first plane of anesthesia. Trichloroethylene is nonexplosive; it is not flammable when mixed with air, but may become so when mixed with oxygen. When the latter mixture is used, there is risk of ignition especially when a cautery is employed. In such instances, admixture with air is preferable. Trichloroethylene is highly stable when stored in closed containers away from light. However, to avoid possible oxidation it is inadvisable to retain any unused portion in an anesthetic machine; such portions should be discarded. Trichloroethylene may be heated without decomposition; but, when its vapor, diluted with air, is exposed to a naked flame, decomposition occurs giving rise to hydrochloric acid and traces of phosgene. The agent or its vapor should not be allowed to come in contact with hot surfaces

BOUND VOLUMES

Journal of the American Pharmaceutical Association

$11.00

Order from the Association

2215 Constitution Avenue, N. W.
Washington 7, D. C.

REHEIS

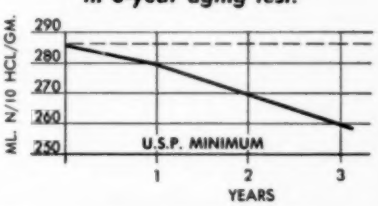
Al (OH)$_3$ DRIED GELS

ACID-CONSUMING CAPACITY

Better than U.S.P. Minimum

EVEN AFTER 3 YEARS

Actual measured values in 3-year aging test:



F-2000 Powdered Gel Aging Test for Acid-Consuming Capacity

At start of test	286
After 1 year	279
After 2 years	270
After 3 years	260

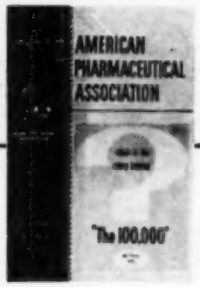
*Conducted according to U.S.P. XIV acid-consuming capacity test procedure

This is just one *more* reason why REHEIS is the standard of quality in dried and compressed Al(OH)$_3$ gels.

FINE REHEIS CHEMICALS

Write for data and samples
...just clip this ad to your signed letterhead.

REHEIS COMPANY, INC.
Manufacturers of Fine Chemicals
BERKELEY HEIGHTS • NEW JERSEY



A Preview of Advertising

In the October 1954 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▶ In two insertions, **Abbott Laboratories** features **Optilets,** "the only therapeutic formula multivitamin tablet," and *filmtab* **Erythrocin Stearate.**

▶ **The American Druggists' Fire Insurance Co.** emphasizes that its contracts are "available only to druggists."

▶ "Therapeutic bile" is the description given by the **Ames Company** to its product **Decholin,** for use in the treatment of hepatobiliary disorders.

▶ The **Chesebrough Mfg. Company's** insertion features its **Vaseline** sterile petrolatum gauze dressings.

▶ More than two decades' use has proved the effectiveness of **Thantis** lozenges—offered by **Hynson, Westcott & Dunning, Inc.**

▶ **Achromycin** "is far and away the leading brand of tetracycline," reports **Lederle Laboratories.**

▶ For use in the treatment of anemia, **Eli Lilly & Company** features **'Trinsicon.'**

▶ The following prescription specialties are listed by the **S. E. Massengill Company** in its announcement: **Adrenosem, Aminodrox, Broniacin, Livitamin, Massengill Powder, Obedrin, Salcedrox,** and **Semhyten.**

▶ **Abdol,** its new vitamin product, is the subject of **Parke, Davis & Company's** announcement.

▶ **Terramycin** and **Bonamine,** says **Pfizer Laboratories,** have the "built-in sell!"

▶ B.Sc., M.Sc. and D.Sc. degree courses are offered by **Philadelphia College of Pharmacy and Science,** "oldest yet most modern of its kind in the Americas."

▶ **A. H. Robins Company** reports that **Donnatal** (tablets, capsules, elixir) exhibits "maximum spasmolytic efficacy and minimum incidence of side effects."

▶ "For greater compounding ease—wider physician use"—**Roerig** suggests **Tetracyn** oral suspension, chocolate flavored.

▶ To relieve cold symptoms and to curb complications, **Schering** advocates **Coricidin** with penicillin.

▶ **Sharp & Dohme** reports that "in pernicious anemia or tropical sprue in relapse," **Redisol** results in "remarkable increase in strength, vigor."

▶ "The high salicylate blood level produced by **Pabirin,**" reports **Smith-Dorsey,** "controls pain and discomfort in the majority of arthritics."

▶ The high sales potential of its **Benzedrex Inhaler** is emphasized by **Smith, Kline & French Laboratories.**

▶ **Cheracol** is publicized by **Upjohn** as "the recognized leader . . . of expectorants and cough preparations."

▶ For use in the treatment of dematoses eczema and pruritus, **U. S. Vitamin Corporation** suggests **Panthoderm** cream.

▶ **Peritrate,** "an effective prophylaxis against angina pectoris," and **Methium,** "the most potent hypotensive agent," are publicized by **Warner-Chilcott.**

▶ **Winthrop-Stearns** stresses that *now* is the time to order **Creamalin** and **Tricreamalate.**

▶ **Wyeth's** new antihypertensive agent is **Ansolysen,** "which presents the advantage of comparative freedom from by-effects."

Look for details in the Practical Pharmacy Edition of This Journal—Out October 20

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIII | *OCTOBER, 1954* | NUMBER 10 CONSECUTIVE NO. 20

The Determination of Inositol as the Hexaacetate*

By F. J. BANDELIN, H. E. DEANE, and R. E. PANKRATZ

The gravimetric determination of inositol as the hexaacetate is described. The method is based upon the reaction of inositol with acetic anhydride to form the water-insoluble, solvent-soluble hexaacetate. The latter is extracted from the reaction mixture with ethylene dichloride after decomposition of the excess acetic anhydride with water. The inositol hexaacetate is weighed after evaporation of the ethylene dichloride. Interference of sugar in the reaction may be overcome by destruction of the sugar through fermentation. By-products of fermentation and other polar materials are removed by passing the fermentation liquid through appropriate ion exchange columns. The method is applicable to tablets and syrups containing inositol. Other lipotropic agents such as choline, betaine, and methionine do not interfere.

INOSITOL is a hexahydroxycyclohexane and is one family of isomers having the same general chemical structure but existing in different spatial configurations. The compound was first isolated by Scherer (1), in 1850, from animal muscle and was named, by him, from the Greek word "inos," meaning muscle. From the circumstances of its occurrence in plants and in animal tissues, it has been variously called dambose, inosite, inosin, phaseomannitol and meat or muscle sugar.

The hexahydroxycyclohexanes, or cyclitols, consist of eight geometric isomers, one of which can also exist in the *d*- or *l*- form, thus making a total of nine stereoisomers. Four of the possible isomers have been found to be naturally occurring (inositol, *d*-inositol, *l*-inositol, and scyllitol) while three others (isoinositol, alloinositol and mucoinositol) have been synthesized and characterized. Seven of the isomers are optically inactive as the result of internal structural compensation while the remaining two (*d*-inositol and *l*-inositol) are optically active.

Through the years the name inositol has come to be used as a generic term for the entire family of isomeric hexahydroxycyclohexanes; and with certain prefixes attached, also for the designation of several additional individual isomers. Thus the isomeric hexahydroxycyclohexanes are sometimes referred to as "the inositols."

To differentiate the compound originally discovered by Scherer, his name of inositol for the isomer has been elaborated, at various times, with the prefixes myo-, meso-, or i- (optically inactive). It is this isomer (obtained commercially by hydrolysis of the calcium-magnesium salt of inositol hexaphosphate "phytin" which is derived from corn and having a melting range of from 223 to 226°) which is the compound of pharmaceutical interest and the item of commerce today known as inositol.

* Received May 1, 1954, from the Control Laboratory, Flint, Eaton and Co., Decatur, Ill.

The emergence of inositol from a laboratory curiosity to a factor in animal and human nutrition and its use as a possible lipotropic agent in medicine has brought about widespread use in pharmaceutical preparations. Despite its general usage, methods of determination have remained a problem. Several microbiological methods have been developed. Among these is the method utilizing the yeast *Saccharomyces cerevisiae* developed by Woolley (2) and by Williams and his co-workers (3, 4). A modification of the method of Atkin, *et al.* (5) for pyridoxine employing *Saccharomyces carlsbergensis* has been described by Gyorgy (6). Another method using a mutant of the mold *Neurospora crassa*, which requires inositol for growth (7), has been reported.

Although several chemical methods for inositol have been described, these leave much to be desired for use as assay methods. In 1929 Fleury and Marque (8) reported a method for the chemical estimation of inositol based on the oxidation of inositol by potassium iodomercurate in alkaline solution. The resulting mercury reacts with standard iodine solution and the excess iodine is titrated with thiosulfate. This method and several modifications (9–11) as well as the periodic acid oxidation method developed by Platt and Glock (12) all have the disadvantage of giving inordinately high blanks in titration. This nonspecific and circuitous approach requires considerable manipulation with the possibility of sizable errors.

Considering the absence of a simple and reliable chemical method for inositol, we investigated the reaction of inositol with acetic anhydride and the isolation and gravimetric estimation of inositol hexaacetate. Sugars interfere with the determination of inositol in that they, like inositol, readily form acetylated derivatives. Since sucrose is a common constituent of liquid pharmaceutical preparations, a method for eliminating sugar interference in the estimation of inositol has been included. Details and results of this investigation and its application to pharmaceutical preparations are given.

EXPERIMENTAL

Reagents.—*Inositol*, optically inactive recrystallized from water-methanol solutions; melting point after drying at 110° is 226°. *Diluted sulfuric acid*. Analytical reagent grade diluted to 10%. *Acetic anhydride*, analytical reagent. *Baker's yeast*, active form commonly available in grocery stores. *Ammonium sulfate*, analytical reagent. *Dowex® 50 Ion Exchange*, 20–50 mesh (2 x 30 cm. column). *Dowex® 1 Ion Exchange Resin*, 20–50 mesh. (2 x 30 cm. column). *Acetic anhydride—dilute sulfuric acid solution;* prepared by adding 1 part of 10% sulfuric acid to 50 parts of acetic anhydride. *Ethylene dichloride*, redistilled commercial grade. *Sodium bicarbonate*, analytical reagent. *Yeast suspension*, 1/4 cake bakers' yeast suspended in water, centrifuged, and liquid decanted. This is repeated until the supernatant solution is clear. This yeast is now suspended in 20 ml. water.

Procedure for Inositol Pure or in Tablet Mixtures in Absence of Sugars or Other Acetylatable Materials.—Weigh precisely 0.2 Gm. of inositol or its equivalent in tablet mixtures into a 250-ml. beaker and add 5 ml. of the acetic anhydride-dilute H_2SO_4 reagent. Cover with a watch glass and heat on a steam bath for twenty minutes.

Remove beaker from steam bath and chill in ice bath before adding approximately 100 ml. water. (Chilling prevents the violent reaction of water with acetic anhydride.) Boil the contents of the beaker until the liquid volume is reduced by half. Cool and transfer to a 250-ml. separatory and extract with four 30-ml. portions ethylene dichloride using the solvent to rinse the original beaker. Wash each portion of solvent with 10 ml. water in a second separatory funnel, filter through cotton, and collect in a tared 125-ml. Soxhlet flask. Evaporate to dryness, drive off any remaining solvent by heating in an oven at 110° for one hour, cool in a desiccator, and weigh as inositol hexaacetate.

The weight of the acetate × 0.4167 represents the inositol in the original sample.

Results obtained with weighed quantities of pure recrystallized inositol (m. p. 226°) are given in Table I.

Results obtained with 0.5-Gm. tablets are given in Table II.

TABLE I.—GRAVIMETRIC DETERMINATION OF INOSITOL AS THE HEXAACETATE

	Inositol Found, %	Deviation, %
	100.02	+0.02
	99.86	−0.14
	99.92	−0.08
	99.88	−0.12
	100.16	+0.16
	100.04	+0.04
	99.86	−0.14
	99.92	−0.08
	100.04	+0.04
	99.84	−0.16
	99.82	−0.08
	100.10	+0.10
	100.00	±0.00
	100.00	±0.00
	99.86	−0.14
Mean	99.95	−0.038

Procedure in Sucrose Solution.—Dilute exactly 15 ml. of the inositol sucrose solution, containing approximately 500 mg. of inositol and not more than 10 Gm. (75% w/v) of sucrose, to 100 ml. in a volumetric flask. Mix well and pipet 10 ml. into a 125-ml. Erlenmeyer flask. Add 0.3 Gm. ammonium sulfate and 5 ml. yeast suspension equivalent to 1/16 cake or roughly 1.0 Gm. and 5 ml. water. Suspend the yeast in the solution by swirling and allow to ferment overnight in an incubator at 37.5°.

TABLE II.—GRAVIMETRIC DETERMINATION OF INOSITOL IN 0.5-GM. TABLETS

Wt. of Sample	Wt. of Hexa-acetate	Inositol, %	Inositol Found per Tablet, Gm.	Theory, %
0.0881	0.1753	82.92	0.4992	99.84
0.0904	0.1800	83.31	0.5016	100.31
0.0953	0.1897	82.96	0.4994	99.88
0.0976	0.1922	83.07	0.5001	100.02
0.1059	0.2112	83.10	0.5003	100.06
0.0991	0.1972	82.93	0.4993	99.86
0.0994	0.1978	82.94	0.4993	99.86
0.0976	0.1942	82.91	0.4991	99.82
0.1193	0.2275	82.91	0.4991	99.82
0.1096	0.2183	83.01	0.4997	99.84

Transfer to a glass-stoppered 35-ml. centrifuge tube and centrifuge down. Decant the clear liquid and wash the yeast with 20 ml. fresh water. Again centrifuge and decant.

Pass the combined fermentation solution and washing through a column of Dowex® 50 ion exchange (hydrogen cycle) and then through a second ion exchange column of Dowex® 1 (hydroxy cycle) and collect in a 400-ml. beaker. Rinse the columns consecutively with sufficient water until 300 ml. are collected in the beaker.

Boil the solution until reduced in volume to 20 ml. and evaporate to dryness on a steam bath.

Add 5 ml. acetic anhydride-dilute H_2SO_4 solution, cover with a watch glass, and warm 20 minutes on the steam bath.

Chill in an ice bath and add 200 ml. water. Boil until reduced in volume by half.

Transfer to a 250-ml. separatory funnel and extract with four 30-ml. portions ethylene dichloride using each to rinse the beaker. Wash each portion through 50 ml. 5% $NaHCO_3$ and 50 ml. water in individual separatory funnels. Filter through cotton into a tared 125-ml. Soxhlet flask. Evaporate to dryness.

To the Soxhlet flask add 15 ml. water and warm on a steam bath twenty minutes. Chill in an ice bath and decant through a coarse, sintered glass filter. Wash the Soxhlet flask with two 5-ml. portions ice water decanting each through the filter. Discard the water.

Rinse the glass filter with successive portions of hot ethylene dichloride, collecting the filtrate in the original tared Soxhlet flask until approximately 100 ml. are collected.

Evaporate to dryness, drive off the remaining solvent by placing in an oven for one hour at 110°, cool in a desiccator, and weigh as inositol hexaacetate.

The weight of the inositol hexaacetate × 0.4167 × 10 = Gm. inositol in 15-ml. sucrose solution.

DISCUSSION

The ratio of 10% H_2SO_4 to acetic anhydride in the acetylating reagents seems to be critical. A greater amount of acid causes charring and less often allows an incomplete reaction.

The twenty minutes acetylation also was the most advantageous condition to prevent the above difficulties.

The chilling of the freshly acetylated solution is imperative to avoid dangerous splattering.

The use of ethylene dichloride as an extraction solvent has an advantage in that it forms an azeotrope with water which boils at a lower temperature than the solvent. By this method the water is removed with the solvent leaving a dry precipitate of inositol hexaacetate.

Ammonium sulfate is added to prevent the *p*H of the fermenting mixture from falling too low, and thus inhibiting complete fermentation of the sugars.

The use of 125-ml. Erlenmeyer flasks for the small volume of fermentation liquid provides a large surface area to facilitate the escape of carbon dioxide formed and thus aid the completion of fermentation.

By passing the fermented solution consecutively through beds of strongly acidic and strongly basic ion exchange resins, most of the remaining fermentation products are removed. However, some of these materials are not sufficiently polar to be held by either resin and these are finally removed by leaching them out of the isolated and dried inositol hexaacetate with water. Warming the water in contact with the inositol hexaacetate promotes the solution of these materials in it and subsequent chilling with ice water reduces the solubility of the hexaacetate. All attempts to remove these extraneous materials immediately after fermentation by solvent extraction of the aqueous solution were unsuccessful.

Other lipotropic agents such as choline, betaine, and methionine may be present in large quantities but do not interfere. Under conditions of the procedure, starch does not interfere.

Inositol hexaacetate obtained through this procedure had a melting range of 212–215° whereas Heilbron (13) states it to be 216°.

SUMMARY

A method for the derermination of inositol by reaction with acetic anhydride, extraction of the inositol hexaacetate formed with ethylene dichloride and gravimetric determination as the hexaacetate, is described. The method is applied to pure inositol and tablet mixtures free from sugars and other acetylatable materials with accurate and reproducible results having a mean deviation of less than 0.05 per cent.

TABLE III.—TYPICAL RESULTS OF DETERMINATIONS OF INOSITOL IN A FLAVORED SYRUP CONTAINING SUCROSE

Inositol in Sucrose Solutions[a]		
Found, Gm./15 ml.	Theory, %	Error, %
0.511	102.2	+2.2
0.496	99.2	−0.8
0.508	101.5	+1.5
0.501	100.2	+0.2
0.488	98.6	−1.4
0.511	102.2	+2.2
0.499	99.8	−0.2
0.495	99.0	−1.0
Mean	100.34	+0.5

[a] Inositol 0.5 Gm./15 ml. in a flavored syrup.

In syrups containing inositol, interference of sugar may be eliminated by fermenting the sugars in the sample with yeast. The fermentation liquid is passed consecutively through a strongly acidic and a strongly basic ion exchange bed to remove fermentation by-products. The inositol is then acetylated and the hexaacetate determined gravimetrically after extraction with ethylene dichloride.

Although the method gives somewhat less accurate and less consistent results in liquid preparations containing sugar, it may nevertheless be considered as an adequate method for the estimation of inositol in sugar-containing liquids.

Other lipotropic agents often used in combination with inositol such as choline, betaine, and methionine do not interfere in the determinations carried out with this procedure.

REFERENCES

(1) Scherer, J., *Ann.*, **73**, 322(1850).
(2) Woolley, D. W., *J. Biol. Chem.*, **140**, 453(1941).
(3) Williams, R. J., Eakin, R. E., and Snell, E. E., *J. Am. Chem. Soc.*, **62**, 1204(1940).
(4) Williams, R. J., Stout, A. K., Mitchell, H. K., and McMahan, J. R., Univ. of Texas Publ. No. 4137, 1941, pp. 27–30.
(5) Atkin, L., Shultz, A. S., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.*, **15**, 141(1943).
(6) Gyorgy, P. "Vitamin Methods," Vol. I, Academic Press, New York, 1950, pp. 251–256, 439–445.
(7) Beadle, G. W., *J. Biol. Chem.*, **156**, 683(1944).
(8) Fleury, P., and Marque, J., *J. pharm. chim.*, **10**, 241 (1929).
(9) Young, L., *Biochem. J.*, **28**, 1428(1934).
(10) Young, L., *ibid.*, **28**, 1435(1934).
(11) Gregory, R. A., *ibid.*, **29**, 2798(1935).
(12) Platt, B. S., and Glock, G. E., *ibid.*, **37**, 709(1943).
(13) Heilbron, I., "Dictionary of Organic Compounds," Oxford University Press, New York, 1946.

A New Partition Chromatographic Procedure for the Assay of Pharmaceuticals*

By DANIEL BANES

A new procedure for the assay of pharmaceuticals is described. The sample is incorporated with an immobile solvent and a supporting medium to form a column suitable for chromatographic development, and the therapeutic substances are then isolated by differential elution. Details are presented for the rapid assay of digitoxin, the quantitative separation of strychnine and quinine, and the analysis of mixtures containing aspirin, phenacetin, and caffeine.

IN THE ANALYSIS of pharmaceuticals it is usually necessary to segregate the therapeutically active substances. The method of "shake-out" extraction by distribution between immiscible solvents has been used extensively for this purpose. However, it becomes tedious when troublesome emulsions are encountered, and it often fails to separate quantitatively even compounds with dissimilar solubilities. Partition chromatography is highly selective, but it requires the careful control of experimental conditions, and it is frequently applicable only to limited quantities of material. A convenient procedure, which combines the selectivity of chromatography with the flexibility of immiscible solvent extraction, has been developed in this laboratory for the assay of many pharmaceuticals. The mechanics of some of these assays are presented here to illustrate the principles of the procedure.

* Received July 1, 1954, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

THE RAPID DETERMINATION OF DIGITOXIN

An accurate method for the analysis of digitoxin tablets, based upon the extraction of the total glycosides and their separation by liquid-liquid partition chromatography, has been described in an earlier report from this laboratory (1). It was shown there that digitalis glycosides are readily soluble in formamide, and that digitoxin can be separated from other digitoxosides by repeated extraction from diluted formamide solution with chloroform-benzene mixtures. These observations

TABLE I.—THE DETERMINATION OF DIGITOXIN

Sample	Description	Digitoxin		Other Digitoxosides	
		Rapid Method, %	Extended Method, %	Rapid Method, %	Extended Method, %
1	Powder	98.2	98.9	1.5	0.8
2	Powder	96.2	97.8	3.9	2.2
3	Powder	63.1	62.6	36.9	37.4
4	Powdered tablets	0.209	0.208	0.014	0.013
		0.214[a]			
		0.214[a]			
5	Powdered tablets	0.190	0.190	0.017	0.016
		0.194[a]			
		0.195[a]			
6	Powdered tablets	0.078	0.079	0.018	0.013
		0.079[a]			
7	Powdered tablets	0.076	0.078	...	...
8	Powdered tablets	0.088	0.092	...	...
9	Powdered tablets	0.181	0.186	...	...
10	Powdered tablets	0.175	0.176	...	...
11	Powdered tablets	0.178	0.176	...	...
12	Powdered tablets	0.083	0.078	...	...
13	Powdered tablets	0.075	0.073	...	...
14	Powdered tablets	0.157	0.161	...	...

[a] Analysis by Jonas Carol, Division of Pharmaceutical Chemistry, U. S. Food and Drug Administration.

suggested the feasibility of a new procedure, in which the sample containing digitoxin is treated with formamide, and the entire mixture is incorporated with a suitable supporting medium to form the immobile phase of a chromatographic column. Digitoxin and other digitoxosides are then separated from each other and from tablet excipients by differential elution.

METHOD OF ANALYSIS

Materials.—*Solvents.*—(*a*) Formamide.—Shake the C. P. grade solvent with anhydrous potassium carbonate for fifteen minutes, and filter. Distill in all-glass apparatus under vacuum. Reject the first portion of distillate containing water, and collect the fraction which boils at about 101°/12 mm. Hg (115°/25 mm. Hg). Store over sulfuric acid until the odor of ammonia is no longer discernible.

(*b*) Benzene, isooctane, chloroform, and alcohol of U. S. P. grade.

Solutions.—(*a*) Benzene-chloroform (2 + 1) and Formamide-water (2 + 1).—Wash about 150 ml. of chloroform four times with 250-ml. portions of water. Filter through paper, and mix 100 ml. of the filtrate with 300 ml. of benzene. Shake the mixture with 20.0 ml. of formamide and 10.0 ml. of water for five minutes, and filter the two solutions separately through paper.

(*b*) Alkaline Picrate Reagent.—Mix 20 ml. of a 1% aqueous solution of picric acid with 10 ml. of 5% sodium hydroxide solution, dilute to 100 ml. with water and mix. The reagent is stable for two or three days.

(*c*) Standard Digitoxin Solution.—Dissolve 20.0 mg. of U. S. P. reference standard digitoxin in alcohol and dilute to exactly 50 ml. with alcohol. Dilute 10.0 ml. of the stock solution to 100 ml. with alcohol.

Diatomaceous Silica Support.—Celite® 545, produced by the Johns-Manville Co.

Chromatographic Trap.—Prepare a chromatographic tube from a 200 x 25-mm. test tube. Fit it with a packing rod, about 400 mm. in length, having a flattened head 22–23 mm. in diameter. Place a small wad of purified cotton in the bottom of the tube.

Wash Layer.—Add about 2 Gm. of Celite® to 1 ml. of water in a 100-ml. beaker. Mix thoroughly with a stirring rod or scoop, until the mixture appears fluffy and uniform, and transfer to the chromatographic tube. Press down lightly with the packing rod. (The wash layer should be 15–20 mm. thick.)

Trap Layer.—Add 3 Gm. of Celite® to 2 ml. of (2 + 1) formamide-water solution in a 150-ml. beaker, mix thoroughly, and transfer to the tube. Press the trap layer down lightly and evenly.

Preparation of the Sample.—Levigate a powdered sample equivalent to 2 mg. of digitoxin with 2.0 ml. of water in a 250-ml. beaker. Add 4.0 ml. of formamide, stir thoroughly, and cover the beaker with a watch glass. Heat the mixture at 50–60° for twenty minutes with frequent stirring. Cool, and add about 7 Gm. of Celite®. Stir thoroughly until the mass appears uniform and does not stick to the beaker. Transfer the mixture quantitatively to the chromatographic trap through a powder funnel in several portions, pressing it down with the stirring rod. Use a rubber policeman to sweep adhering particles from the beaker and funnel into the tube. Scrub the beaker and stirring rod with about one Gm. of Celite®, and add the dry washings to the tube through the funnel. Repeat the washing with two further portions of Celite®. Place a wad of purified cotton in the tube and press it down on the column with the packing rod, sweeping the Celite® on the sides of the tube before it. (The overall height of the column should be 120–150 mm.)

Separation of Digitoxin by Differential Elution.—Moisten the column with about 30 ml. of isooctane. Use gentle air pressure, if necessary, to speed the passage of the solvent down the column. Wash with an additional 120 ml. of isooctane. (A stoppered, long-stemmed separator serves as a convenient reservoir.) When the isooctane has percolated through the column, elute the digitoxin with 190 ml. of benzene-chloroform solution, collecting the

eluate in a 200-ml. volumetric flask. Wash the stem of the tube with a stream of chloroform, dilute to exactly 200 ml. with chloroform, and mix.

Colorimetric Determination of Digitoxin.—Remove duplicate 20-ml. aliquots of the eluate to small Erlenmeyer flasks, and evaporate to dryness on the steam bath, with the aid of a current of air. Moisten the residues with about 0.5 ml. of alcohol, and again evaporate to dryness. Add 5.0 ml. of alcohol to the cooled flasks, stopper, and let stand fifteen minutes with occasional shaking. Transfer duplicate 5.0 ml. aliquots of the standard digitoxin solution to small flasks, and prepare a 5-ml. alcohol blank. Add 3.0 ml. of the alkaline picrate reagent to the blank, to a sample solution, and to a standard, and mix by swirling. Protect the mixtures from intense light. After ten minutes, determine the absorbances of the standard and sample solutions relative to the blank at 495 mμ, repeating the measurements at two-minute intervals until the maximum values are attained. Repeat the process for the duplicate sample and standard aliquots, and compute the digitoxin content of the sample.

RESULTS OF ANALYSIS

A series of fourteen preparations were analyzed for digitoxin by the extended method previously published, and by the present rapid method of differential elution. Results of these analyses are presented in Table I. In several cases (Samples 1–6), more polar digitoxosides were eluted with 150 ml. of chloroform, and determined as gitoxin by the Keller-Kiliani reaction (2). The data indicate that the rapid method yields reproducible values and is highly reliable for the elution of both digitoxin and other digitoxosides. The digitoxin fractions were obtained as crystalline white residues on evaporation from benzene-chloroform solution containing isooctane, and any accurate colorimetric test could be used for its determination. The modification of the U. S. P. XIV test for digitoxin (3) employed here is convenient and sensitive, and the colors obey Beer's law. Application of the Keller-Kiliani test for digitoxose could be used to detect the presence of aglucones in the digitoxin fraction.

The proposed determination of digitoxin can be completed in little more than three hours, and the attention of the operator is required only a third of that time. It is unnecessary to maintain strictly specified conditions of temperature, flow-rate, or composition of solvents.

THE SEPARATION OF STRYCHNINE AND QUININE

Haddock and Evers (4) have published a procedure for the separation of strychnine and quinine in elixirs, based upon the relative solubilities of the alkaloidal hydrochlorides in chloroform and in dilute hydrochloric acid. The reliability of their extraction procedure has been questioned by Herd (5) and others. Strychnine hydrochloride can be separated quantitatively from quinine hydrochloride by applying the method of differential elutions to the system suggested by Haddock and Evers.

TABLE II.—RECOVERIES OF STRYCHNINE

Sample	Description	Strychnine Recovered, %
15	1.32 mg. strychnine sulfate	101.3
16	1.32 mg. strychnine sulfate plus 25.8 mg. quinine hydrochloride	98.5
17	1.06 mg. strychnine plus 51.6 mg. quinine hydrochloride	98.4
18	1.75 mg. strychnine phosphate plus 100 mg. quinine hydrobromide	98.6
19	10 ml. of iron, quinine, and strychnine elixir, N.F. IX	100.4
20	10 ml. of iron, quinine, and strychnine phosphates elixir, N.F. IX	100.4

METHOD OF ANALYSIS

Chromatographic Column.—Prepare a chromatographic tube packed with a wash layer, 1 ml. of water adsorbed on 1 Gm. of Celite®, and a trap layer 3 ml. of 2 *N* hydrochloric acid adsorbed on 3 Gm. of Celite®. Evaporate 10 ml. of the elixir in a 250-ml. beaker to a volume of 3–4 ml. Add 2 ml. of 6 *N* hydrochloric acid, cool, and mix thoroughly with about 6 Gm. of Celite®. Transfer the sample layer to the chromatographic tube without loss, as directed above under *Digitoxin*.

Elution and Determination of Strychnine.—Moisten the column with about 25 ml. of water-washed ether, and permit 100 ml. of the solvent to percolate through the column. Elute the strychnine hydrochloride with 150 ml. of freshly prepared water-washed chloroform, collecting the eluate in a beaker. Evaporate carefully to dryness with the aid of a current of air. Dissolve the residue in 5 ml. of warm 2 *N* hydrochloric acid, cool, and dilute to exactly 100 ml. Compare the absorbance of this solution at 256 mμ with that of a standard containing 2 mg. of strychnine in 100 ml. of 0.1 *N* hydrochloric acid solution. Compute the strychnine content of the elixir.

Determination of Quinine.—Add 15 ml. of water to the chromatographic tube, force the fluid down the tube with gentle air pressure, and collect the effluent liquid in a separator. Repeat with five 15-ml. portions of water. Separate the chloroform and wash it in a second separator containing 20 ml. of water. Combine the aqueous layers, dilute with water to 200 ml., and determine quinine in the presence of iron by the method of Carol (6).

RESULTS OF ANALYSIS

The proposed method was first applied to 5-ml. aliquots of solutions containing various strychnine and quinine salts in 2 *N* hydrochloric acid, to test the efficacy of the separation procedure. (Samples 15–18 in Table II.) Samples of iron, quinine and strychnine elixir, and iron, quinine and strychnine phosphates elixir were also assayed. Recoveries of strychnine ranged from 98.4 to 101.2%. Quinine was determined only for the elixir samples and recoveries were 101.7% and 102.5% of the true values. All of the strychnine hydrochloride residues were white and crystalline, and the ultraviolet absorp-

tion spectra of their acid solutions exhibited the characteristic peak near 256 mμ, and the minimum near 228 mμ. Absorbances between 300 and 350 mμ were negligible (absence of quinine). A solution containing 100 mg. of quinine hydrochloride and no strychnine yielded an eluate identical with that obtained in applying the procedure to 5 ml. of 2 *N* hydrochloric acid.

THE ANALYSIS OF MIXTURES CONTAINING ACETYLSALICYLIC ACID, PHENACETIN, AND CAFFEINE

The method in the A. O. A. C. *Official Methods of Analysis* (7) for the analysis of mixtures containing acetylsalicylic acid, phenacetin, and caffeine has been criticized by Holt (8). He suggested that acetylsalicylic acid and phenacetin be separated from caffeine by extracting a suspension of the sample in dilute hydrochloric acid with ether, to avoid the time-consuming hydrolysis of phenacetin required in the older method. However, it has been shown (8, 9) that neither phenacetin nor caffeine is obtained in a pure condition in Holt's rapid procedure. Phenacetin and caffeine can be separated quantitatively by means of differential elution techniques after incorporation of the mixture in a chromatographic column. Smith (10) has described a similar elution procedure.

METHOD OF ANALYSIS

Isolate the acetylsalicylic acid by the A. O. A. C. method and estimate by any reliable procedure. Dilute the combined chloroform extracts to exactly 200 ml. Transfer an aliquot containing not more than 65 mg. of phenacetin to a 250-ml. beaker, and evaporate to dryness. Add 4.0 ml. of formamide and 2.0 ml. of water, cover with a watch glass, and warm on the steam bath to dissolve the residue. Add 6 Gm. of Celite® and mix thoroughly. Transfer without loss to a chromatographic tube containing 1 ml. of water adsorbed on 1 Gm. of Celite® as wash layer and 5 ml. of 2 *N* HCl adsorbed on 5 Gm. of Celite® as trap layer. Cover the trap layer with about one gram of Celite® to separate the acid from the diluted formamide.

Phenacetin.—Moisten the column with 25 ml. of water-washed ether and elute phenacetin with an additional 120-ml. of the solvent. Evaporate the eluate to dryness with the aid of a current of air. Determine the phenacetin in the sample gravimetrically, or spectrophotometrically at 251 mμ by comparing the absorbance of the residue, dissolved in a suitable volume of alcohol, with that of a solution containing 1 mg. of pure phenacetin in 100 ml. of alcohol.

Caffeine.—Elute caffeine from the column with 150 ml. of freshly prepared water-washed chloroform, collecting the eluate in a 200-ml. volumetric flask. Add 2 ml. of alcohol and dilute to exactly 200 ml. with chloroform. Evaporate an aliquot containing about 1 mg. of caffeine. Dissolve the residue in 5 ml. of 2 *N* hydrochloric acid, and dilute to exactly 100 ml. with water. Compare the absorbance of this solution at 272 mμ with that of a solution containing 1 mg. of pure caffeine in 100 ml. of 0.1 *N* hydrochloric acid. Compute the caffeine content of the sample.

RESULTS OF ANALYSIS

Table III includes the results of analysis for phenacetin and caffeine by the proposed method of separation, and by a modification designed to accommodate the column to dried samples containing more than 100 mg. of phenacetin. These samples (Samples 25–27 in Table III) were warmed with 10 ml. of ether, and then mixed with 5 ml. of water and 7 Gm. of Celite® to form the sample layer. The wash layer and trap layer were the same as those described in the proposed method. Samples 26 and 27 were treated chromatographically without the prior extraction of acetylsalicylic acid. The ether eluates were permitted to percolate through an alkaline trap (5 ml. of molar tripotassium phosphate adsorbed on 5 Gm. of Celite®) for the removal of the aspirin. The spectral properties of the eluted substances indicated a quantitative partition of caffeine and phenacetin, and satisfactory recoveries for both drugs.

DISCUSSION

Incorporation of the dosage form of the sample in a column suitable for chromatographic development may be employed in the isolation of any substance

TABLE III.—THE DETERMINATION OF PHENACETIN AND CAFFEINE

Sample	Description	Caffeine Recovered, %	Phenacetin Recovered, %
21	15 mg. caffeine	100.0	...
22	62 mg. phenacetin	...	100.0
23	4.25 mg. caffeine plus 31.6 mg. phenacetin	100.0	99.5
24	Tablet mixture extract containing 4.25 mg. caffeine and 31.6 mg. phenacetin	102.2	101.3
25	31.8 mg. caffeine plus 316 mg. phenacetin plus 460 mg. acetyl salicylic acid	99.1	100.8
26	Tablet mixture containing 72.6 mg. caffeine, 281.2 mg. phenacetin and 223.5 mg. acetylsalicylic acid	99.2	99.1
27	Tablet mixture containing 17.3 mg. caffeine, 318.8 mg. phenacetin and 310 mg. acetylsalicylic acid.	100.2	98.4

which is easily soluble in an immobile phase solvent, and can be eluted with a convenient mobile solvent. The method of differential elution affords a means of obtaining pure substances from such a column in sufficient quantities to permit the application of independent tests for identification and determination. These techniques have been employed in the assay of estrogenic preparations, barbiturates, cardioactive glycosides, and mixed sulfonamides. They should prove of value also in the analysis of food extracts, blood, urine, and other biological fluids.

SUMMARY

1. A new procedure for the assay of pharmaceuticals has been described.

2. Details have been presented for the application of the procedure to the rapid determination of digitoxin, the separation of quinine and strychnine, and the analysis of mixtures containing acetylsalicylic acid, phenacetin, and caffeine.

REFERENCES

(1) Banes, D., and Carol, J., THIS JOURNAL, **42**, 674 (1953).
(2) Rowson, J. M., *J. Pharm. Pharmacol.*, **4**, 814(1952).
(3) "United States Pharmacopeia," 14th rev., Mack Publishing Co., Easton, Pa., 1950, p. 181.
(4) Haddock, L. A., and Evers, N., *Quart. J. Pharm. Pharmacol.*, **4**, 314(1931).
(5) Herd, R. L., THIS JOURNAL, **31**, 9(1942).
(6) Carol, J., *J. Assoc. Offic. Agr. Chemists*, **25**, 524(1942).
(7) "Official Methods of Analysis of the Association of Official Agricultural Chemists," 7th ed., Washington, D. C., 1950, p. 580, sec. 32.85.
(8) Holt, K. E., THIS JOURNAL, **35**, 71(1946).
(9) Daoust, R. A., *ibid.*, **42**, 744(1953).
(10) Smith, G., *J. Assoc. Offic. Agr. Chemists*, **37**, 677 (1954).

Sabadilla Alkaloids*

IV. Separation of Veratridine and Cevadine by Partition Chromatography

By GLENN R. SVOBODA and LLOYD M. PARKS

Veratridine and cevadine have been separated in a high state of purity from prepared mixtures of the known alkaloids, from commercial veratrine, and from the mixture of alkaloids obtained by Skellysolve® B extraction of sabadilla seed by partition chromatography on a silicic acid column buffered at *p*H 4.00 and 4.25, using chloroform as the eluting solvent.

IN PREVIOUS WORK on the separation of the sabadilla alkaloids (1–3) difficulty has been encountered in attempts to separate veratridine and cevadine. Fractional crystallization from a suitable solvent was not satisfactory; adsorption chromatography with activated alumina, calcium carbonate or magnesium oxide was unsuccessful (4); and partition chromatography at *p*H of 6, 7, and 8 was likewise unsatisfactory (4) for the reason, as shown here, that these *p*H ranges were too high.

The objective of the present work was to explore further the applicability of partition chromatography to the separation of veratridine and cevadine. To determine the combination of solvent system and buffer under which a suitable separation might reasonably be expected, the partition coefficients of the individual pure alkaloids were determined for various systems at different *p*H values. From these results, it was possible to prepare partition columns on which good separations of veratridine and cevadine could be achieved on both small scale and large scale.

Using chloroform as the eluent and columns buffered at *p*H 4.00 or 4.25 veratridine and cevadine were separated from prepared mixtures of

* Received January 18, 1954, from the School of Pharmacy, University of Wisconsin, Madison, Wisconsin.
This investigation was supported in part by a research grant, H-1083, from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service, and in part by the Research Committee of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

the two alkaloids. Under the same conditions veratridine and cevadine were separated in a fairly high state of purity from commercial veratrine and from the mixture of alkaloids obtained by Skellysolve® B extraction of sabadilla seed.

EXPERIMENTAL

Materials.—Commercial veratrine was obtained from S. B. Penick and Company.

Extraction of sabadilla seed with Skellysolve® B was carried out and the extract processed in the manner previously described (1) to yield, from 10.12 Kg. of seed, 140 Gm. of crude alkaloid mixture, referred to hereafter as Fraction *A*. The ultraviolet absorption of this fraction indicated the presence of veratridine and cevadine (3).

Preparation of Veratridine and Cevadine.—The method of Blount (5) was used for the isolation of veratridine and cevadine from both veratrine and Fraction *A*. The cevadine was further purified by recrystallization from alcohol and veratridine was purified by repeated recrystallization of both the sulfate and perchlorate salts, the increase in purity being determined by the ultraviolet absorption of the free base. In this manner there was obtained cevadine with m. p. 208–211°, and veratridine with extinction coefficient (Ex 100) at 262 mμ consistently above 1700 (2).

Determination of Partition Coefficients.—Two-tenths per cent (w/v) solutions of veratridine and cevadine were prepared in pure benzene and pure chloroform and in benzene and chloroform each containing 2.5%, 5.0%, and 10% (v/v) of absolute ethanol, respectively, making a total of eight different solutions of each alkaloid in the organic solvents.

Two sets of buffer solutions were employed, namely, 0.5 *M* phosphate ranging from *p*H 5.80 to 7.50 and citrate ranging from *p*H 4.00 to 5.50. Buffer solutions were made by mixing solutions of mono- and disodium phosphate and solutions of citric acid and sodium citrate, respectively, checked on the Beckman *p*H meter.

Ten-cc. portions of the buffer and the alkaloid solution were shaken for thirty seconds in 30-cc. separatory funnels and, after separation of the layers, the amount of alkaloid in the organic layer was determined by titration of a 5-cc. aliquot with 0.0254 *N* perchloric acid in glacial acetic acid, using 0.1% crystal violet as indicator (6). Titrations were carried out in 15-cc. centrifuge tubes using a 1-ml. Gilmont Ultramicro buret.

With the chloroform and chloroform-alcohol solutions the end point used was the change from violet to blue. Since this change was not distinct with the benzene and benzene-alcohol solutions the end point used for the latter was the change from blue to blue-green. Applicability and accuracy of the titrations were checked on known solutions containing 10 mg. of each alkaloid. Titration error was 2.8% for veratridine and 6.2% for cevadine.

No attempt was made at temperature control and the room temperature varied from 24 to 26°.

TABLE I.—PARTITION COEFFICIENTS OF VERATRIDINE AND CEVADINE IN CHLOROFORM AND CHLOROFORM-ALCOHOL SYSTEMS

*p*H of Buffer Layer	Partition Coefficient in Organic Layer							
	100% $CHCl_3$		2.5% Alcohol		5% Alcohol		10% Alcohol	
	Ver.	Cev.	Ver.	Cev.	Ver.	Cev.	Ver.	Cev.
4.00	1.08	0.33	1.15	0.36	1.16	0.36	1.11	0.31
4.25	1.56	0.50	1.65	0.56	1.72	0.51	1.74	0.51
4.50	2.83	1.03	2.94	1.18	3.22	1.06	3.08	1.07
4.75	5.25	2.42	4.71	3.05	5.62	2.92	7.00	2.97
5.00	5.71	3.31	5.33	3.69	8.09	4.56	10.11	3.93
5.25	7.93	5.17	6.46	7.40	10.11	11.50	22.26	6.81
5.50	8.71	7.77	7.85	11.50	14.63	32.33	39.00	10.63

Results of the determination of the partition coefficients are shown in Tables I and II, and plots of the log of the partition coefficient *vs. p*H for both alkaloids in pure chloroform and pure benzene are shown in Figs. 1 and 2. From these results it may

TABLE II.—PARTITION COEFFICIENTS OF VERATRIDINE AND CEVADINE IN BENZENE AND BENZENE-ALCOHOL SYSTEMS

*p*H of Buffer Layer	100% Benzene		2.5% Alcohol		5% Alcohol		10% Alcohol	
	Ver.	Cev.	Ver.	Cev.	Ver.	Cev.	Ver.	Cev.
5.80	0.62	0.54	0.80	0.48	0.81	0.68	0.64	0.79
6.00	0.80	0.71	0.97	0.75	0.90	0.90	0.93	1.02
6.25	1.18	..	1.30	1.16	1.29	1.44	1.37	1.80
6.50	1.98	2.34	2.33	2.34	2.53	2.96	2.91	5.33
6.75	2.83	4.43	3.48	4.71	4.43	5.76	5.54	14.63
7.00	3.31	5.89	4.29	6.87	5.41	8.43	5.74	32.33
7.25	3.67	6.94	4.59	8.71	6.46	12.33	8.52	32.33
7.50	3.67	8.90	5.85	12.70	6.87	75.92	9.99	75.92

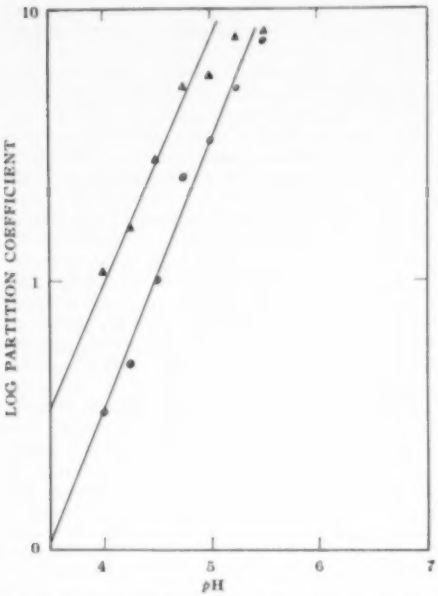


Fig. 1.—Log of partition coefficient *vs.* *p*H for cevadine ⊙ and veratridine △ in 100% chloroform.

be seen that the partition coefficients of the two alkaloids are very similar in benzene. The intersection of the plots in the benzene systems may be due to some type of association of one of the alkaloids with the solvent. Plots for the other solvent systems employed were similar to those shown here, i. e., parallel in the chloroform-alcohol systems and intersecting in the benzene-alcohol systems.

These results show that the partition coefficients of the two alkaloids are sufficiently different in chloroform systems to allow good separation by partition procedures. Varying amounts of alcohol in the chloroform appeared to have little effect on the partition coefficients. Figure 1 indicates that the best separation of veratridine and cevadine should be achieved by partitioning the mixture between chloroform and a buffer at *p*H 4.25 (7).

Partition Chromatography.—Partition columns were prepared using 15 Gm. of silicic acid (Mallinckrodt, 100 mesh) and 15 cc. of buffer in tubes with an inside diameter of 1.2 cm. When packed in the usual way (3) the columns had a height of 30 cm. The flow rates of such columns were varied, by tightness of the packing, from 6 to 28 drops per minute. Three hundred milligram samples were used for all runs of veratrine while the prepared mixtures of known veratridine and cevadine contained 100 mg. of each alkaloid. The eluates were collected in 5-cc. fractions in test tubes and were then washed into tared aluminum dishes for evaporation and weighing.

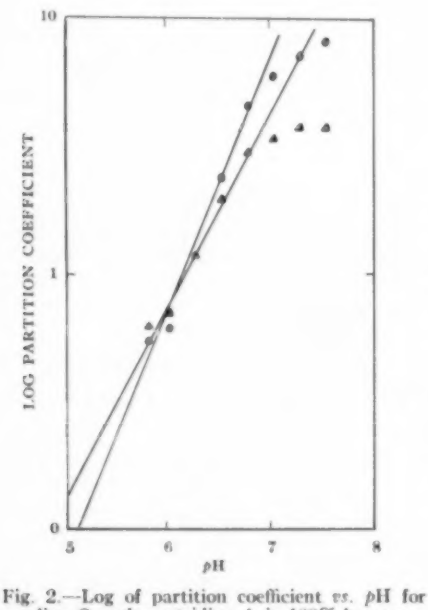


Fig. 2.—Log of partition coefficient *vs.* *p*H for cevadine ⊙ and veratridine △ in 100% benzene.

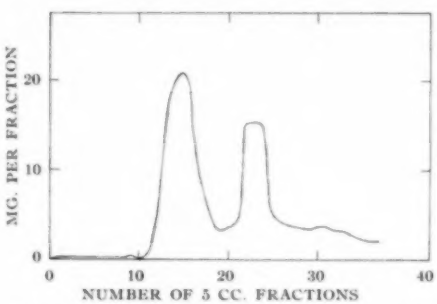


Fig. 3.—Partition chromatography of prepared mixture of veratridine and cevadine at *p*H 4.25; flow rate of 6 drops per minute.

Runs were made at *p*H 4.00, 4.25, and 4.50, using reagent grade chloroform as eluant in all but one case which is noted below. Total recovery of veratridine plus cevadine from veratrine samples averaged 80% while recovery from the prepared mixtures of known veratridine and cevadine averaged 96%. Identity of the alkaloids and purity of those found in the peak fractions were established by ultraviolet absorption and by titration as noted below.

Figure 3 shows the results obtained on a typical run with a prepared mixture of known veratridine and cevadine at *p*H 4.25. The extinction coefficient (Ex 100) at 262 mμ for the veratridine obtained in the first peak (fraction 15) was 1780 while the cevadine obtained in the second peak showed a typical absorption spectrum (2). Results obtained on runs with veratrine were similar to those shown in Fig. 3.

Figure 4 shows the results obtained on a typical run with a prepared mixture of known veratridine and cevadine at *p*H 4.00. The extinction coefficient (Ex 100) at 262 mμ for the veratridine obtained in fraction 16 was 1720 while the cevadine obtained

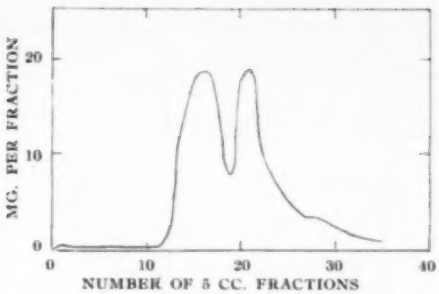


Fig. 4.—Partition chromatography of prepared mixture of veratridine and cevadine at *p*H 4.00; flow rate is 28 drops per minute.

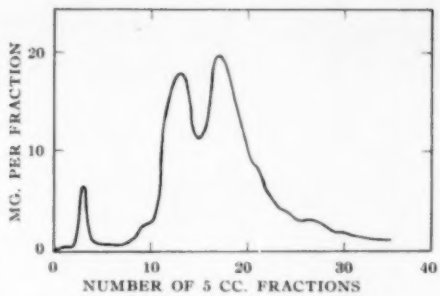


Fig. 5.—Partition chromatography of Fraction *A* at *p*H 4.00.

from fraction 21 showed typical absorption. Results obtained on runs with veratrine under the same conditions were similar to those shown in Fig. 4.

Since the partition coefficients indicated that the rate of elution of the alkaloids would be higher at *p*H 4.50 it was thought that partition chromatography at this higher *p*H would cut down the tailing of the cevadine portion to some extent. This was found to be the case on columns run at *p*H 4.50 but the separation of veratridine and cevadine was not as sharp as on the columns which were run at *p*H 4.00 or 4.25.

In another attempt to improve the separation, columns were run at *p*H 4.25 using chloroform-Skellysolve® B (9:1) as the eluant with the thought that the reduced polarity of such a solvent would slow down the rate of elution and thus spread the distance between the veratridine and cevadine peaks. Under these conditions, however, no separation was obtained and the alkaloids were eluted together in one peak fraction.

As a preliminary to large scale separation of veratridine and cevadine from Fraction *A* a run was made on a 300-mg. sample of Fraction *A* at *p*H 4.00 using chloroform as the eluant, with the results shown in Fig. 5. The first small peak (fraction 3) consisted of highly colored material. The extinction coefficient (Ex 100) at 262 mμ for the veratridine obtained in fraction 13 was 1820.

Large Scale Separation of Fraction A.—Partition columns were prepared using 750 Gm. of silicic acid and 750 cc. of buffer at *p*H 4.00 in tubes with an inside diameter of 5.1 cm. and runs were made with 15-Gm. samples of Fraction *A*, collecting thirty-five 250-cc. fractions. Results of a typical run under these conditions are shown in Fig. 6. Fractions 11 to 15, comprising the first peak, weighed 3.013 Gm. (veratridine) and fractions 16 to 27, comprising the second peak, weighed 5.17 Gm. (cevadine). Each of these combined fractions was rerun under the same conditions with the results of combined fractions 11 to 15 (veratridine) shown in Fig. 7 and the results of combined fractions 16 to 27 (cevadine) shown in Fig. 8.

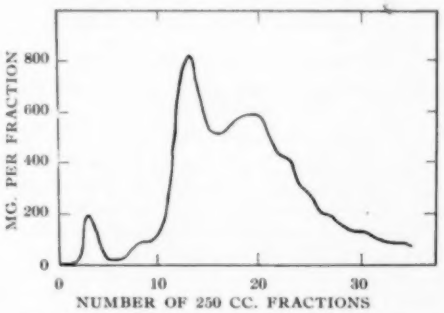


Fig. 6.—Large scale partition chromatography of Fraction *A* at *p*H 4.00.

The eluted veratridine from this rerun was purified by repeated recrystallization of its sulfate and then converted to the free base to yield 0.38 Gm. of veratridine with Ex 100 at 262 mμ of 1938. A considerable amount of the alkaloid was lost during recrystallization of the sulfate.

The eluted cevadine from the rerun (fractions 20 to 30 of Fig. 8) was recrystallized from alcohol to yield 0.6 Gm. of product, m. p. 212.5–214.5°. An

TABLE III.—TITRATION OF VERATRIDINE FROM PEAK CHROMATOGRAPHIC FRACTIONS

Veratridine Obtained from	Veratridine Determined by: Weight,[a] mg.	Ex 100 262 mμ	Titration,[b] mg.	Ex 100, 262 mμ
Veratrine at *p*H 4.25	18.3	1586	15.65	1853
Veratrine at *p*H 4.00	19.0	1745	14.0	2179
Veratrine at *p*H 4.50	22.5	1650	18.8	1975
Prepd. mixture at *p*H 4.00	18.9	1718	14.65	2075
Veratrine by Blount (5)	50.0	1850	44.6	2072

[a] Higher values by weight may be due in part to incomplete removal of solvent.
[b] Titration values were reproducible to ± 0.0001 cc.

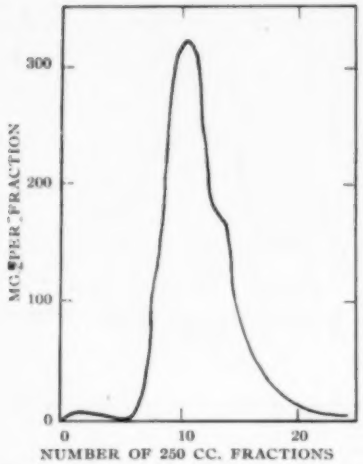


Fig. 7.—Rerun of veratridine fractions from Fig. 6 at *p*H 4.00.

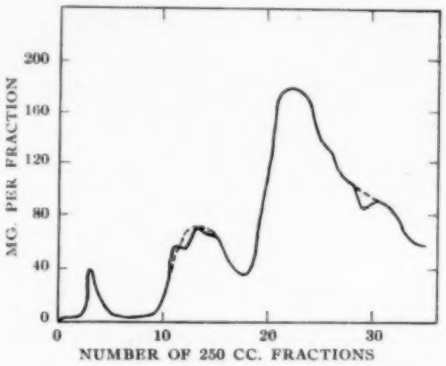


Fig. 8.—Rerun of cevadine fractions from Fig. 6 at *p*H 4.00.

additional 1.1 Gm. of cevadine, m. p. 212–213°, was obtained by stripping the column with absolute methanol and recrystallization from alcohol-water.

Titration of Veratridine from Peak Fractions.—As a further means of checking identity and purity of the alkaloids separated by partition chromatography, titrations were carried out on samples of veratridine found in the peak fractions of several runs. Aliquots of the samples prepared for ultraviolet absorption analysis were placed in 15-cc. centrifuge tubes, the solvent evaporated, and the residue dissolved in chloroform and titrated in the same manner as was done in determining the partition coefficients. Ultraviolet absorption measurements were made on other aliquots of the same samples and from these the extinction coefficients at 262 mμ were calculated, using both the concentrations found by weighing the alkaloid in each peak fraction and those found by titration. For comparison purposes a sample of the purest veratridine obtained by Blount's method (5) was also titrated. Comparison of the results, shown in Table III, shows that a one-step partition chromatography of either veratrine or a prepared mixture of known veratridine and cevadine results in a separation of the two alkaloids in a state of purity which compares favorably with the procedure used by Blount.

REFERENCES

(1) Poetsch, C. E., and Parks, L. M., THIS JOURNAL, **38**, 522(1949).
(2) Poetsch, C. E., Higuchi, T., and Parks, L. M., *ibid.*, **38**, 525(1949).
(3) Hennig, A. J., Higuchi, T., and Parks, L. M., *ibid.*, **40**, 168(1951).
(4) Lach, J. L., M. S. Thesis, University of Wisconsin 1952.
(5) Blount, B. K., *J. Chem. Soc.*, **1935**, 122.
(6) Fritz, J. S., "Acid-Base Titrations in Non-Aqueous Solvents," G. Frederick Smith Chemical Company, Columbus, Ohio, 1952.
(7) Weissberger, A., "Technique of Organic Chemistry," Vol. III, Interscience Publishers, Inc., New York, 1950, p. 200.

WHO MAKES IT?

The registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals:

Truxillic acid
Chroman-5,6-quinone
4-Nitrohydrindene
Mucinase
Cyclital
p-Nitrophenol-β-D-glucoside
Hydroxytetronic acid
Reductone
Dibromogallic acid
n-Ethyl glycocyamine
Elemicin
Chlorocarvacrol
1,2,3-Butanetriol
Ethyl α-methylacetoacetate
Oxaloacetonitrile
α,α′-Diphenyl-β-picryl hydrazil
Potassium thioplatinate
Monoiodotyrosine
N,N-Diethylthioformamide
d-Mannosaccharo-1,4:3,6-dilactone

Analysis of Mixtures of Theobromine and Caffeine by Spectrophotometric Method*

By JAMES W. MILES† and DUANE T. ENGLIS

Acidic solutions of both caffeine and theobromine exhibit absorption spectra of similar nature and magnitude with maxima near 273 mμ. If the solutions are made basic there is little change in absorption for the caffeine but the theobromine shows a considerable increase in absorption in the region of 220–254 mμ. For each constituent, the concentration is proportional to absorbancy for a given set of conditions. In mixtures, the amount of theobromine can be calculated directly from the difference in absorbancy observed for the basic and acidic solutions at 240 mμ. Knowing the amount of theobromine, its absorbancy in acid solution at 273 mμ may be calculated and the residual absorption observed gives a measure of the amount of caffeine present. As an alternate procedure, the quantities of each may be calculated by the solution of simultaneous equations involving the absorbancies at 240 and 273 mμ in basic solution. Representative experimental data and results for the mixtures of varying proportions in a total concentration of 30 mg. per liter are shown.

THEOBROMINE AND caffeine occur together in cocoa nibs and allied products and also in the process of methylation of theobromine to produce caffeine. Consequently, methods for the analysis of such mixtures are of interest and value. An ultraviolet spectrophotometric method has been proposed by Ishler, Finucane, and Borker (1) for the determination of caffeine in coffee and Jones and Thatcher (2) have made a similar application to medicinals. No methods have appeared for the spectrophotometric determination of theobromine alone or in mixtures with caffeine. In the analysis of mixtures of organic materials, advantage may frequently be taken of the fact that one constituent is subject to a considerable change in the magnitude of some physical property as a result of chemical or physical action, while others are not affected. One well-known example of such a phenomenon is the change in rotation of sucrose resulting from its hydrolysis to invert sugar, which change is employed for its quantitative estimation. It was believed that a somewhat similar principle involving ultraviolet absorbance might be employed for the determination of theobromine in the presence of caffeine. The two compounds are very similar in structure, differing only in that caffeine is completely substituted while theobromine has a hydrogen in place of one methyl group on the caffeine. Hence, under certain conditions, one might anticipate that their ultraviolet absorption spectra would be very similar. However, by virtue of the presence of the hydrogen instead of the methyl group, theobromine can undergo enolization. Consequently it was believed that it would show a marked change in structural nature and corresponding absorption as a result of a change in *p*H. The purpose of this paper was to study the ultraviolet absorption characteristics of theobromine and caffeine under different conditions with the hope that a simple method could be devised for the determination of each constituent without a separation of one from the other.

* Received February 11, 1954, from the Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Ill.

† Present address: College of Pharmacy, University of Kentucky, Louisville, Ky.

EXPERIMENTAL AND RESULTS

Apparatus.—All absorption curves and absorption measurements were made with a Cary Model 11 recording spectrophotometer.

Purification of Caffeine and Theobromine.—The alkaloids used throughout the experiment were purified by sublimation. This was accomplished very easily for the caffeine by placing a few grams of U. S. P. grade caffeine in an evaporating dish covered with a watch glass. The dish was warmed on a hot plate and the fine sublimed crystals collected on the watch glass. The crystals were removed and dried in an oven at 110°. The sublimation process was more difficult for the theobromine than for the caffeine. Crystals of N. F. grade theobromine were sublimed by placing them in a closed system and applying vacuum. The system was heated and the sublimed crystals collected on a cold finger projecting into the apparatus. These crystals were also dried at 110° and stored in a desiccator.

Study of Absorption Characteristics.—A stock solution of caffeine was prepared by dissolving exactly 100 mg. of the pure alkaloid in warm water and making to a volume of 1 L. Twenty-five milliliter portions of this solution were transferred to two 100-ml. volumetric flasks. One was made to volume with 0.1 *N* NaOH and the other with 0.1 *N* HCl. Portions of these solutions were placed in 1-cm. quartz cells and their ultraviolet absorption spectra recorded. Solutions of theobromine were prepared in exactly the same manner and their spectra recorded. The resulting curves are shown in Figs. 1 and 2.

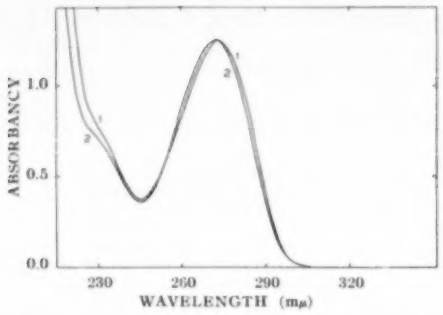


Fig. 1.—Ultraviolet absorption spectra of caffeine in aqueous solutions. (*1*) 25 mg. per liter in 0.1 *N* NaOH; (*2*) 25 mg. per liter in 0.1 *N* HCl.

It may be noted that alkaline solutions of theobromine exhibit a considerable increase in absorbancy over acidic solutions. On the other hand, alkaline solutions of caffeine show little difference in absorbancy from the acidic solutions. Therefore, if a sample contained both caffeine and theobromine, it appeared possible to determine the theobromine content by measuring the increase in absorbancy of its alkaline solutions over the corresponding acidic solutions. The total alkaloids could then be determined by measuring the absorbancy of the acidic solution at 273 mμ. This method would require the preparation of working curves for both caffeine and theobromine. It may also be observed from Fig. 2 that it would be possible to determine the two alkaloids simultaneously in alkaline solution provided $A_{1\text{ cm.}}^{1\text{ mg./L}}$ values were established at two appropriate wave lengths and the proper simultaneous equations employed. These procedures were subjected to test.

Method Employing Working Curves.—Solutions of pure caffeine and pure theobromine were prepared in concentrations from 10 mg. per liter to 30 mg. per liter. Two solutions of each concentration were prepared, one was made in 0.1 *N* HCl and the other in 0.1 *N* NaOH. The absorbancy of each acid solution was measured at 273 mμ and at 240 mμ. For each alkaline solution the absorbancy was measured only at 240 mμ. The wavelength 240 mμ was chosen for making the latter readings because this represented the point of greatest change in absorbancy of the theobromine solutions when going from acid to alkaline media. Also the change in absorbancy for the caffeine solutions in going from acid to alkaline media was least at this wavelength. From these data, the increase in absorbancy of the alkaline solutions over the acid solutions at 240 mμ was calculated for each concentration of both caffeine and theobromine solutions. These data are tabulated in Tables I and II.

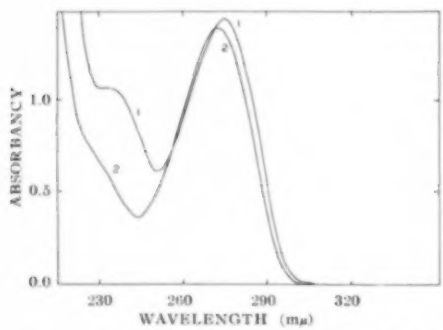


Fig. 2.—Ultraviolet absorption spectra of theobromine in aqueous solutions. (*1*) 25 mg. per liter in 0.1 *N* NaOH; (*2*) 25 mg. per liter in 0.1 *N* HCl.

TABLE I.—ABSORBANCY OF THEOBROMINE SOLUTIONS (1-CM. CELLS)

Conc., mg./L.	*A* (at 273 mμ) in HCl	(*a*) *A* (at 240 mμ) in NaOH	(*b*) *A* (at 240 mμ) in HCl	(*a* − *b*) ΔA
10	0.55	0.37	0.16	0.21
15	0.83	0.56	0.24	0.32
20	1.10	0.73	0.32	0.41
25	1.38	0.92	0.38	0.54
30	1.65	1.10	0.47	0.63

TABLE II.—ABSORBANCY OF CAFFEINE SOLUTIONS (1-CM. CELLS)

Conc., mg./L.	*A* (at 273 mμ) in HCl	(*a*) *A* (at 240 mμ) in NaOH	(*b*) *A* (at 240 mμ) in HCl	(*a* − *b*) ΔA
10	0.49	0.18	0.17	0.01
15	0.73	0.25	0.25	0.00
20	0.98	0.34	0.33	0.01
25	1.23	0.43	0.41	0.02
30	1.48	0.50	0.48	0.02

Excellent conformity to Beer's law was shown by both the caffeine and theobromine solutions when the absorbancies of the acid solutions at 273 mμ were plotted against concentration. When the change in absorbancy at 240 mμ, ΔA, was plotted against concentration, straight lines were also obtained. The slope of the resulting curve for caffeine was very slight since alkaline solutions of caffeine exhibited absorbancies only slightly higher than corresponding acidic solutions.

A series of synthetic sample solutions were prepared containing known mixtures of caffeine and theobromine. Two aliquots were withdrawn from each sample. One was made to a given volume with 0.1 *N* HCl and the other was made to the same volume with 0.1 *N* NaOH. The absorbancy of each acid solution was observed at 240 and 273 mμ and the absorbancy of each basic solution at 240 mμ. A first approximation of the theobromine content was made by subtracting the absorbancy of the acid solution at 240 mμ from the absorbancy of the corresponding alkaline solution at 240 mμ and referring to the working curve (ΔA *vs.* theobromine concentration). Having determined the theobromine concentration, the caffeine was then determined by referring to the working curves of the acid solutions of theobromine and caffeine at 273 mμ. In some cases where the caffeine concentration was high, a slight correction in the first approximation of theobromine was made. This correction was obtained from the ΔA curve for caffeine. The results of the analyses of mixtures using the calibration curves are given in Table III.

Method Employing Simultaneous Equations.—If simultaneous equations are employed in the analysis of the mixtures, it is necessary to measure the absorbancy at two wavelengths in the basic medium only. $A_1^{1\ \frac{mg.}{cm.}/L.}$ values for each component were established at the two wavelengths for the purpose of deriving the necessary equations. These were calculated from the data in Table I and II. The following results were obtained.

At 273 mμ, $A_1^{1\ \frac{mg.}{cm.}/L.}$
for theobromine = 0.0569
for caffeine = 0.0480
At 240 mμ, $A_1^{1\ \frac{mg.}{cm.}/L.}$
for theobromine = 0.0364
for caffeine = 0.0171

In order to solve by simultaneous equations, let

x = mg. of theobromine per liter
y = mg. of caffeine per liter
A_m = absorbancy of mixtures at 273 mμ
A_n = absorbancy of mixtures at 240 mμ

Then,

$$x(0.0569) + y(0.0480) = A_m$$
$$x(0.0364) + y(0.0171) = A_n$$

And

$$x = \frac{(48.0)(A_n) - (17.1)(A_m)}{0.774}$$

or

$$x = 62.0\,A_n - 22.1\,A_m$$

Solving for y,

$$y = \frac{36.4\,A_m - 56.9\,A_n}{0.774}$$

or

$$y = 47.0\,A_m - 73.5\,A_n$$

The same seven mixtures which were analyzed by the use of calibration curves were analyzed again by the use of the above equations and the results compiled in Table IV.

DISCUSSION

It may be noted in general that the accuracy by one method is about the same as by the other. As is to be expected, the percentage errors are greatest when the amount of either alkaloid is small. The reliability of the working curve method depends mainly upon the magnitude of the difference between the absorbancy of the theobromine in alkaline and acid solution at 240 mμ; in the simultaneous equation method, it depends mainly upon the difference between the absorption of the theobromine and caffeine when both are examined in alkaline solution at this wavelength. Because the slopes of the curves are quite steep in the region of 240 mμ, the wavelength selection at this point is particularly critical. An absorbancy value taken at a wavelength only slightly removed from the prescribed position would contribute much to the error in the determination. The simultaneous equation method has one distinct advantage over the working curve method in that it is necessary to examine the solutions only in the alkaline medium. Although the results by neither method give a highly accurate evaluation of either of the individual components, a close approximation of each is obtained. The accuracy of the estimation of the total alkaloids by either method is excellent—the average error being ±1.0% using calibration curves, and ±1.6% using the simultaneous equations. A more precise method involving the separation and determination of each component will be the subject of another paper. Although higher accuracy can be attained by first separating the two substances, the time required is much greater.

TABLE III.—ANALYSIS OF THEOBROMINE-CAFFEINE MIXTURE. USE OF WORKING CURVES INVOLVING THEIR DIFFERENTIAL ABSORPTION IN ACID AND ALKALINE SOLUTIONS

Theobromine Taken, mg./L.	Caffeine Taken, mg./L.	A[a]	A_t[b]	Theobromine Found, mg./L.	Caffeine Found, mg./L.	Total Alkaloid Found, mg./L.
25	5	0.52	1.62	24.8	5.2	30.0
25	5	0.55	1.60	26.2	3.2	29.4
20	10	0.42	1.59	19.6	10.6	30.2
20	10	0.42	1.60	19.4	11.0	30.4
15	15	0.32	1.55	14.8	15.0	29.8
10	20	0.21	1.54	9.6	20.8	30.4
10	20	0.21	1.54	9.6	20.8	30.4

[a] A—Difference in absorbancy of the alkaline and acid solutions measured at 240 mμ.
[b] At—Total absorbancy of the acid solution at 273 mμ.

TABLE IV.—ANALYSIS OF MIXTURES OF THEOBROMINE AND CAFFEINE BY SIMULTANEOUS EQUATIONS

Theobromine Taken, mg./L.	Caffeine Taken, mg./L.	—Absorbance of the Mixture— A_m at 273 mμ	A_n at 240 mμ	Theobromine found mg./L.	Caffeine found mg./L.	Total Alkaloid found mg./L.
25	5	1.65	0.99	24.9	4.8	29.7
25	5	1.65	0.99	24.9	4.8	29.7
20	10	1.60	0.90	20.4	9.0	29.4
20	10	1.62	0.89	19.4	10.7	30.1
15	15	1.56	0.82	16.4	13.1	29.5
10	20	1.56	0.68	7.7	23 3	31.0
10	20	1.54	0.68	8.2	22.4	30.6

SUMMARY

1. The ultraviolet absorption characteristics of theobromine and caffeine in both acidic and alkaline solutions have been investigated.

2. Methods have been developed for the simultaneous determination of the two constituents in mixtures containing up to 30 mg. per liter of the total alkaloids.

3. Data for representative analyses of mixtures in which the quantities of the two constituents are varied within a total of 30 mg. per liter are given.

REFERENCES

(1) Ishler, N. H., Finucane, T. P., and Borker, E., *Anal. Chem.*, **20**, 1162(1948).

(2) Jones, M., and Thatcher, R. L., *ibid.*, **23**, 957(1951).

Blood Sugar Levels and Urinary Excretion of Intravenously-Infused Glucose, Fructose, Galactose, and Sorbitol*

By VICTOR P. SEEBERG, BARBARA WHITNEY, and DOROTHY GOLDMAN

Groups of rabbits were infused intravenously with glucose, fructose, galactose, and sorbitol at a constant rate and total dose approximating the usual clinical rate and total dose of parenterally administered sugars. Urinary excretion was least with glucose and increased in the following order: fructose, sorbitol, and galactose. Though not always statistically significant, differences in the blood sugar curves were also found. Particularly interesting was a marked elevation of blood reducing sugar during infusion with sorbitol. The possible clinical and biochemical significance of the findings are discussed.

FOR MANY YEARS, glucose has been the standard carbohydrate for parenteral alimentation. Recently, considerable interest has been shown in other sugars, particularly fructose. Among the advantages proposed for fructose in this use are greater retention and utilization. Weichselbaum, Elman and Lund (1) reported lower total blood sugar levels and less urinary excretion in man with intravenous infusions of fructose as compared to glucose. However, Beal, *et al.* (2), found little difference in blood sugar levels in man after intravenous infusion of similar quantities of the two hexoses, and in contrast to the results of Weichselbaum's group, found greater quantities of reducing substances in the urine after fructose than after glucose. Smith, *et al.* (3), have also reported greater excretion of fructose than glucose following intravenous infusion of the respective carbohydrates.

An examination of the experimental procedure of the different investigators reveals considerable variation in the conditions of the experiments. Weichselbaum's subjects were infused with 1,000 ml. of 10 per cent solutions of each of the two sugars at rates varying from 0.6 to 2.0 Gm. per Kg. per hour. Part of Beal's subjects received like quantities in two and one-half to three and one-half hour periods, but the weights of only a few of the patients are stated. Assuming an average body weight of 70 Kg., these infusions were given at rates of 0.4 to 0.5 Gm. per Kg. per hour. In another series he gave 3,000 ml. of 10 per cent solutions of the sugars during a twenty-four hour period, but the actual infusion time is not given, and again the patients' weights are not stated. Smith used a constant infusion rate of 1.0 Gm. per Kg. per hour, but infused for a total period of only thirty minutes.

It appeared possible that conflicting reports on blood serum levels and urinary excretion of glu-

* Received April 10, 1954, from the Research Division, Cutter Laboratories, Berkeley, Calif.

cose and fructose in man were due in part to the varying conditions of administration used by the different investigators and the uncontrollable variables inherent in a small clinical series. It was therefore deemed desirable to investigate these points by infusing the two sugars into animals under carefully controlled conditions, by use of a rate and total dose approximating the usual clinical rate and total dose on a body weight basis.

In addition to glucose and fructose, the hexose galactose and the hexahydric alcohol sorbitol were also included in the study. Pieskow and Weirzuchowski (4) reported that galactose was utilized only one-third as well as glucose or fructose when infused in dogs at the rapid rate of 2 Gm. per Kg. per hour for three-hour periods. Reports on sorbitol vary. Richardson (5) administered 100 ml. and 200 ml. of 50 per cent solutions of sorbitol to humans at the very rapid rate of 8 to 10 ml. per minute. The sorbitol disappeared from the blood in two hours. Average urinary excretion was reported to be 15 per cent of the administered dose. Todd, *et al.* (6), found amounts up to 50 per cent in the urine when 50 ml. of a 50 per cent solution was administered to dogs over a five-minute period. No reports could be found on the utilization and excretion of galactose or sorbitol when infused at rates approximating those usually used in parenteral carbohydrate administration.

EXPERIMENTAL

Procedure.—Male albino rabbits were used as the experimental animal. They were routinely fasted overnight before the day of the experiment, and were used only once. The animals were restrained on an animal board for the infusions; no anesthetic was administered. Blood samples were obtained by heart puncture. Urine samples were obtained by means of a stationary catheter inserted into the bladder at the time the first urine sample was drawn. The withdrawal of each urine sample, by means of a syringe, was followed by a water wash which was also withdrawn and combined with the sample. A Brewer automatic pipet equipped with a 1-ml. tuberculin syringe was used to provide the infusion stream. The rate was first adjusted for each animal and then rechecked at 5, 10, 30, 60, and 90 minutes by noting the amount leaving a calibrated reservoir. Each carbohydrate was administered as a 10% solution in pyrogen-free distilled water.

Determination of Blood and Urine Values.—The copper reduction titrimetric method of Somogyi (7) was used in the analysis of blood and urine for reducing sugars. Since the reaction of Somogyi reagent with galactose gives a different titration factor than with glucose or levulose, an approximation was made for total blood sugar in the animals infused with galactose. The normal blood sugar level (glucose) of the animal was determined before starting the infusion and was assumed to remain constant throughout the experimental period. Additional sugar was calculated on the basis of the galactose factor. Sorbitol determinations were made by the modified Hagedorn-Jensen method described by Todd, *et al.* (8). The effect of the presence of reducing sugars on the sorbitol determination was corrected for by a procedure also suggested by Todd. Recovery curves for added sorbitol were established in the presence of 100 mg.% of glucose in blood and urine. In practice, the level of reducing sugar in each sample was determined by the Somogyi method, and then the sample was adjusted to contain the equivalent of 100 mg. % of glucose before subjecting it to the assay for sorbitol.

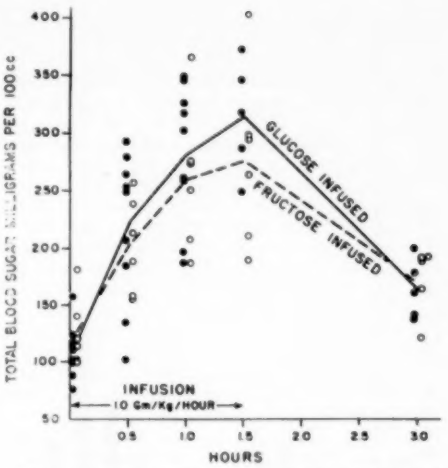


Fig. 1.—Total blood sugar values during and following intravenous infusions of glucose and fructose in rabbits.

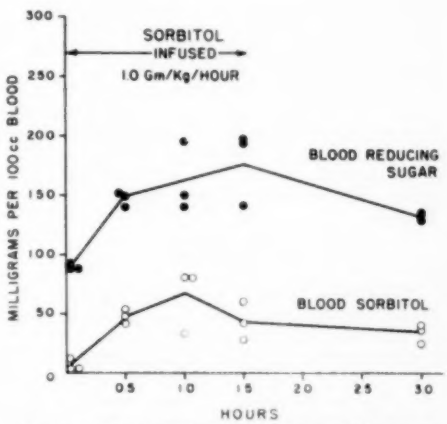


Fig. 2.—Blood sorbitol and blood reducing sugar values during and following intravenous infusions of sorbitol in rabbits.

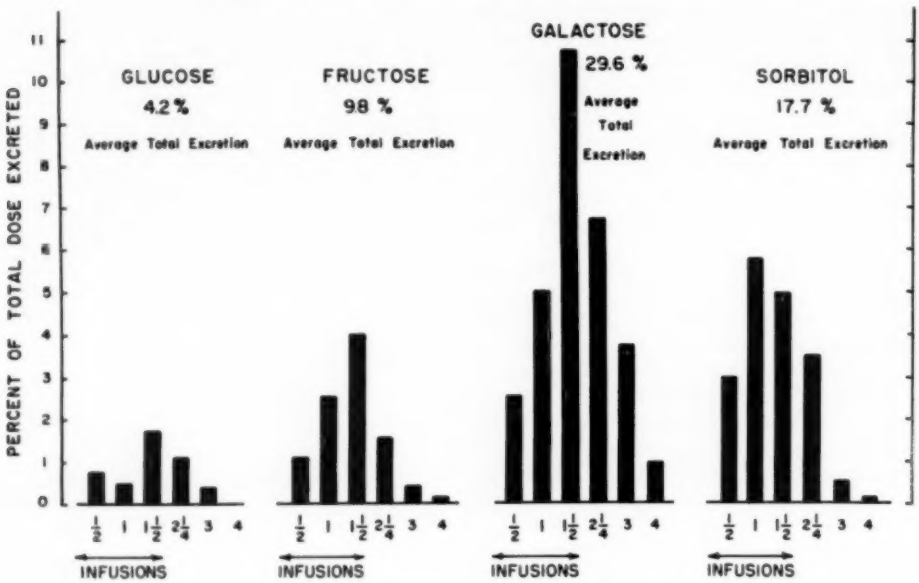


Fig. 3.—Average total urinary excretion and average excretion at various intervals during and following intravenous infusions of glucose, fructose, galactose, and sorbitol at the rate of 1 Gm./Kg./hour.

Rate of Infusion and Total Dose.—Woodyatt (9) found the utilization rate of glucose in man, rabbit, and the dog to be 0.85 Gm./hour. Lockhart and Elman (10) have shown that the maximum rate at which glucose can be administered with no glycosurea is 0.5 Gm./Kg./hour. However, it is well known that glucose is frequently infused clinically at somewhat more rapid rates in order to minimize the period of nursing attention and with full cognizance that in so doing a small percentage is probably lost in the urine. It is claimed that fructose can be given at twice the rate of glucose and with better utilization (11) although the work of Beal (2) and Smith (3) would dispute this statement. In adult patients, 1,000 ml. total doses are common for parenteral sugar solutions. Based on a 10% solution and a patient weight of 70 Kg., this is a total dose of 1.5 Gm./Kg. After a consideration of these experimental factors as well as known clinical practices, a rate of 1 Gm./Kg/hour and a total dose of 1.5 Gm./Kg. were selected for the study as representing a moderately rapid rate of infusion.

RESULTS

Blood Sugar Levels.—The total blood sugar values shown in Fig. 1 indicate that somewhat higher mean values were obtained during the glucose infusion than during the fructose infusion. At the end of one hour of infusion the mean total blood sugar values per 100 ml. of blood were 282 mg. for the rabbits infused with glucose and 260 mg. for the rabbits infused with fructose. At the end of the one and one-half hour infusion period, the values were 315 mg. for the glucose-infused rabbits and 276 mg. for the fructose-infused rabbits. However, because of the overlap of individual values in the two groups, the differences between the means are not significant. At the three-hour period (one and one-half hours after stopping the infusion) blood sugar values had fallen considerably, but were still above original levels. Mean total blood sugar values were 153 mg. for the glucose-infused rabbits and 171 mg. for the fructose-infused rabbits.

The highest blood sugar levels were obtained in the rabbits infused with galactose. The mean total blood sugar value in six infusions at the end of the one and one-half hour infusion period was 341 mg. This is not significantly higher than the mean of 315 mg. of the glucose-infused rabbits but approaches significance over the mean value of 276 mg. of the fructose-infused animals ($P = 0.1$). A mean value of 219 mg. obtained at the three-hour period in the galactose-infused rabbits is significantly higher than the means of the other groups at the same period.

Sorbitol Infusion.—The blood sorbitol levels attained were surprisingly low. However, a rapid and marked rise in blood reducing sugars occurred during the sorbitol infusion. The changing relationships of these values are shown in Fig. 2. It is clear that sorbitol is being rapidly converted to a reducing substance, the identity of which was not determined. In two of the three experimental animals, an actual fall in the blood sorbitol level occurred between the one and the one and one-half hour intervals, even though infusion of sorbitol was continuing. At the three-hour period the blood reducing sugars were still elevated; blood sorbitol levels had fallen to about one-half of their peak values.

Urinary Excretion.—Average total excretion and average excretion during various periods for each group are given in Fig. 3. The average total excretion of 4.2% in the dextrose-infused animals is significantly less than the average of 9.8% total excretion in the levulose-infused animals ($P = 0.02$) In fact, there was only one overlap in the individual values of 9 glucose infusions and 6 fructose infusions. Although blood sugars at the three-hour period were approximately equal in both groups, there were only traces of reducing substances detected in the fourth-hour urines of the glucose-infused group, while measurable quantities were excreted in the fructose-infused group during this period, indicating a lower renal threshold for fructose.

Urinary excretion was greatest during the galactose infusion. As may be seen in the bar graph, considerable excretion continued during the fourth hour. Since urine collection was stopped at that time, the average figure of 29.6% total excretion for galactose may be a little low.

Urinary loss during the sorbitol infusion was also considerable, although excretion had practically ceased by the fourth hour. The excretion consisted of approximately three-fourths sorbitol and one-fourth reducing substances calculated as fructose.

DISCUSSION

In 1915 Woodyatt, *et al.* (9), reported the tolerance limit for fructose intravenously in man, dog, and rabbit to be about one-sixth that of glucose. His average figures were 0.85 Gm./Kg./hour for glucose and 0.15 Gm./Kg./hour for fructose. It has since been shown by Lockhart and Elman (10) that his figure for glucose is a little high, and that rates above 0.5 Gm./Kg./hour are not tolerated in man without some glucose appearing in the urine. However, significantly greater urinary excretion with fructose at equal administration rates in our controlled animal infusions, as well as in the infusions of Beal, *et al.* (2), and Smith, *et al.* (3), in man, only serve to prove that Woodyatt's early comparison of the two sugars, though admittedly crude, was basically sound. There seems little doubt that if the tolerance limit for fructose was also to be established accurately in an experiment similar to that of Lockhart and Elman on glucose, it would come out significantly lower than their figure for glucose.

Lower blood sugar levels have been claimed as evidence that fructose is utilized more rapidly than glucose. It is true that we found a tendency toward lower average blood sugar levels with fructose. However, there was always considerable overlapping of values at any one time interval during and following the infusion so that at no point were the differences significant in our series. Furthermore, about twice as much fructose as glucose was being excreted in the urine, which may partially account for the tendency toward slightly lower blood sugar levels with fructose.

Our results with galactose appear to be a confirmation of results in the older literature, with perhaps the contribution of some comparative quantitation under conditions comparable to clinical infusion. Woodyatt reported a tolerance limit of only 0.1 Gm./Kg./hour for galactose. Both the high total blood sugar levels and the high urinary excretion values in our experiments point to a low utilization rate and a low renal threshold for this sugar. Considering the additional excretion that probably took place after our last urine sample, we estimate that about one-third of this sugar is lost in the urine under the conditions of our infusions.

Although only a few experiments were done with sorbitol, some interesting questions are posed by the results. Elevation of blood reducing sugar in dogs after a single large dose intravenously has been described previously by Todd, *et al.* (6). The rapid rise of blood reducing sugar in our sorbitol infusions is a confirmation of this finding. Todd's group refers to the increase as representing increased blood glucose. From experiments in which sorbitol was perfused through the livers of previously phlorizinized dogs, Embden and Greisbach (12) first suggested that fructose rather than glucose might be the initial oxidation product of sorbitol. This view has recently received additional support by the experiments of Wick, *et al.* (13), on comparative oxidation rates of fructose, glucose, and sorbitol in the alloxan-diabetic rat. To our knowledge, no one has shown conclusively whether the immediate increase in reducing substances in the blood following intravenous sorbitol represents conversion to fructose or glucose, or possibly other reducing substances. It is also interesting to note that at any interval during the infusions with sorbitol, the sum of the blood reducing sugar and the blood sorbitol is lower than the average total blood sugar levels following infusion with the other sugars. Johnson and Deuel (14) have reported that in the fasted rat somewhat more glycogen accumulates in the liver after sorbitol than after glucose administration. The factor of urinary excretion must also be taken into consideration, but it is possible that our observation of lower total blood carbohydrate levels during the sorbitol infusions also indicates a more rapid overall rate of utilization for intravenously infused sorbitol.

It has been reported that sorbitol is not reabsorbed by the kidney tubules (15). This probably accounts for the greater urinary excretion we found during its infusion as compared to the other sugars. Also of interest is the finding that at the three-hour period (one and one-half hours after stopping the infusion) sorbitol blood values as measured by the method used, still showed an average level of 34 mg./100 ml., whereas very little urinary excretion was found during the fourth hour. This finding suggests conversion to a substance giving a positive sorbitol test but behaving differently from sorbitol in the kidney.

SUMMARY

1. Using a moderately rapid rate of infusion in rabbits, urinary excretion of three hexoses and a hexahydric alcohol were as follows: glucose 4.2 per cent, fructose 9.8 per cent, galactose 29.6 per cent and sorbitol 17.7 per cent.

2. A tendency toward somewhat lower total blood sugar values was noted during the fructose infusions as compared to the glucose infusions.

The differences in the mean values were not significant at any time period. Greater urinary excretion may partially account for the tendency toward lower blood sugar levels with fructose.

3. Galactose infusion produced the highest and most persistent blood sugar levels. This, together with high urinary excretion values, confirms a low utilization rate and a low renal threshold for this sugar.

4. Sorbitol infusion produced surprisingly low blood sorbitol levels; instead, a marked elevation of blood reducing sugars occurred. It is clear that sorbitol is rapidly converted to a reducing substance, the identity of which was not determined. Urinary loss during the sorbitol infusion consisted of approximately three-fourths sorbitol and one-fourth reducing substances calculated as fructose.

REFERENCES

(1) Weichselbaum, T. E., Elman, R., and Lund, R. H., *Proc. Soc. Exptl. Biol. Med.*, **75**, 816(1950).
(2) Beal, J. M., Smith, J. L., and Frost, P. M., *Surgery*, **33**, 721(1953).
(3) Smith, L. H., Ettinger, R. H., and Seligson, D., *J. Clin. Invest.*, **32**, 273(1953).
(4) Pieskow, W., and Weirzuchowski, M., *Compt. rend. soc. biol.*, **103**, 415(1930).
(5) Richardson, H. L., Kennedy, J. C., and West, E. S., *Northwest Med.*, **42**, 80(1943).
(6) Todd, W. R., Meyers, J., and West, E. S., *J. Biol. Chem.*, **127**, 275(1939).
(7) Somogyi, M., *ibid.*, **160**, 61(1945).
(8) Todd, W. R., Vreeland, J., Meyers, J. and West, E. S., *ibid.*, **127**, 269 (1939).
(9) Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assoc.*, **65**, 2067(1915).
(10) Lockhart, C. E., and Elman, R., *Surg. Gynecol. Obstet.*, **88**, 97(1949).
(11) Council on Pharmacy and Chemistry of the Am. Med. Assoc., *J. Am. Med. Assoc.*, **153**, 274(1953).
(12) Embden, G., and Griesbach, W., *Z. physiol. Chem.*, **91**, 251(1914).
(13) Wick, A. N., Toshika, N. M., and Barnet, H. N., Meetings Western Soc. Clinical Research, January 29, 1954.
(14) Johnson, C., and Deuel, H. J., *J. Biol. Chem.*, **149**, 117(1943).
(15) Smith, W. W., Finkelstein, N., and Smith, H. W., *J. Biol. Chem.*, **135**, 231(1940).

The Physics of Tablet Compression*

IV. Relationship of Ejection, and Upper and Lower Punch Forces during Compressional Process: Application of Measurements to Comparison of Tablet Lubricants

By EINO NELSON†, S. M. NAQVI, L. W. BUSSE, and TAKERU HIGUCHI

The relationship of the upper and lower punch forces and the ejection force during the process of tablet compression has been studied. Large differences may exist between the maximum force exerted by the upper punch and the maximum force obtained at the lower punch. When such differences exist, substantial forces are needed to eject tablets from the die. The magnitude of the difference between the maximum upper and lower punch force and the magnitude of the ejection force appear to be linearly dependent on the area of the tablet in contact with the die wall. The value of the ejection force and the difference between the upper and lower punch forces are both markedly affected by the presence of lubricants. With a well-lubricated granulation the force exerted by the upper punch is essentially the same as that transmitted to the lower punch and a negligible force is required to eject the tablets from the die. A quantitative method for evaluating lubricants on the basis of these observations is suggested.

RECENTLY, we described an instrumented single punch tablet machine yielding continuous recordings of several compressional variables (1). In the preliminary operation of this machine, it was found that a large differential could exist between the force exerted by the upper punch and the force transmitted to the lower punch during tablet formation. When such large differences existed, a substantial amount of force was necessary to eject the tablet from the die.

The present study was undertaken to: (*a*) investigate the relation between the upper punch, lower punch, and ejection forces in the compres-

* Received March 1, 1954, from the School of Pharmacy, University of Wisconsin, Madison.
Supported in part by grants from Parke, Davis and Company of Detroit, Michigan; Smith, Kline and French Laboratories of Philadelphia, Pa.; and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.
† Parke, Davis and Company Fellow.

TABLE I.—MATERIALS COMPRESSED INTO TABLETS

Substance	Weight of Die Fill, mg.	Remarks
Sulfathiazole granulation[a]	380	Sieved 10–20 mesh
Lactose	368	U. S. P. powder
Sodium chloride	855	Crystals passing 20 mesh
Potassium chloride	553	Crystals passing 20 mesh
Lithium chloride	522	Crystals passing 20 mesh
Ammonium chloride	557	Crystals passing 20 mesh
Sodium iodide	1486	Crystals passing 20 mesh
Potassium iodide	948	Crystals passing 20 mesh
Ammonium iodide	829	Crystals passing 20 mesh
Lithium bromide	1094	Crystals passing 20 mesh
Ammonium bromide	974	Crystals passing 20 mesh

[a] The sulfathiazole granulation was made from sulfathiazole powder and 10% starch paste. The granulation consisted of 97.5% sulfathiazole and 2.5% starch.

TABLE II.—SUBSTANCE TESTED AS LUBRICANTS FOR SULFATHIAZOLE GRANULATION[a]

Substance	Affinity for Granulation	Remarks
Acetophenetidin	Poor	Unsieved powder
Aspirin	Fair	Unsieved powder
Boric acid	Poor	Unsieved powder
Calcium stearate	Good	Unsieved powder
Carbowax® 4000	Poor	Powdered with the aid of dry ice; then sieved through 100 mesh.
Magnesium stearate	Good	Unsieved powder
Sodium stearate	Good	Unsieved powder
Spermaceti	Poor	See Carbowax® 4000
Talc	Good	Unsieved powder
Veegum	Poor	Sieved 100 mesh
Zinc stearate	Good	Unsieved powder

[a] Lubricant concentrations used were 0.5%, 1%, and 2% by weight of granulation.

sion of a sulfathiazole granulation, lactose, and several salts; (*b*) determine the effect of tablet lubricants on the relation between upper and lower punch force and ejection force; and to (*c*) develop a quantitative method for evaluating tablet lubricants.

EXPERIMENTAL

In this work, the instrumented machine (1) was operated with a $^3/_8$ inch flat-headed punch and die set. The compression time in all cases was between one-half and two seconds. The machine was adjusted so that 800 Kg. or more of force was usually transmitted to the lower punch.

Table I lists the various materials compressed. They were weighed to the nearest milligram and stored in desiccators until used. The materials

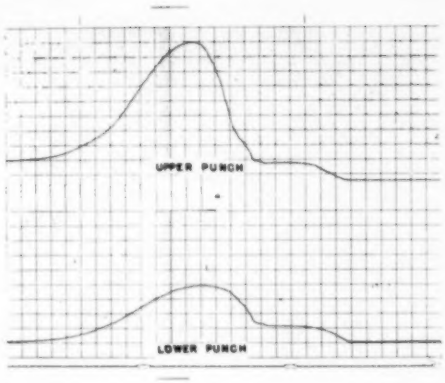


Fig. 1.—Compression of 380 mg. of a sulfathiazole granulation without lubricant added. Level portion above base line to the right of lower punch force curve is ejection force measuring point.

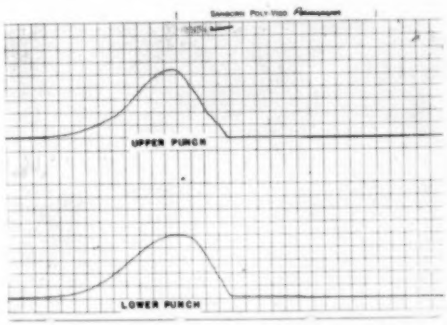


Fig. 2.—Compression of 380 mg. of a sulfathizaole granulation lubricated with 2% calcium stearate. *Cf.* Fig. 1.

tested as lubricants, listed in Table II, were mixed with the sulfathiazole granulation for quantitative evaluation of their effectiveness. These materials were stirred into the granulation with a small brush to coat the sulfathiazole particles. The affinity of a lubricant for the granulation was observed qualitatively by mixing the materials on black weighing paper. In preparation for compression of lubricated samples the machine was adjusted so that 800 Kg. of force was transmitted to the lower punch with unlubricated granulation and left unchanged.

After each compression, in all the compressions made, the die and punches were scrubbed first with dry cotton and then with cotton saturated with acetone.

No attempt was made to control the humidity of the environment during compressions. No runs were made, however, when the relative humidity exceeded 80%.

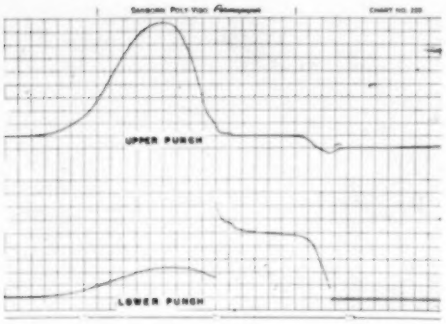

UPPER PUNCH
LOWER PUNCH
EACH CHART DIVISION = 1 MM.
UPPER PUNCH 1 MM. = 40 KGM. FORCE.
LOWER PUNCH 1 MM. = 67 KGM. FORCE.
LOWER PUNCH, EJECTION FORCE
REGION 1 MM. = 13 KGM. FORCE.

Fig. 3.—Compression of Lactose. The sensitivity of the amplifier for lower punch force was increased so that a more accurate reading of ejection force could be obtained. *Cf.* Fig. 1.

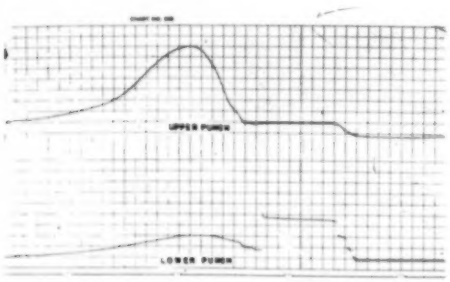

UPPER PUNCH
LOWER PUNCH
EACH CHART DIVISION = 1 MM.
UPPER PUNCH 1 MM. = 40 KGM. FORCE.
LOWER PUNCH 1 MM. = 67 KGM. FORCE.
LOWER PUNCH, EJECTION FORCE
REGION 1 MM. = 13 KGM. FORCE.

Fig. 4.—Compression of sodium iodide crystals.

RESULTS AND DISCUSSION

Typical oscillographic records of the forces involved during tablet compression are shown in Figs. 1–4. In each case the upper and lower curves are the force-time record for the upper and lower punches, respectively. Figure 5 is a calibration curve for the upper punch forces; the lower punch forces, being recorded linearly, are available directly from the oscillographic records. The positive slope side of these curves indicates the increase in force to maximum as the tablet is being compressed. The ejection force may be read from the height of the level portion on the right hand side of the lower punch force-time curve before the zero portion of the curve as has been previously described (1). In Fig. 1, for example, the ejection force is recorded as slightly over 5 mm. on the chart or about 210 Kg. of force.

A comparison of the magnitude of the upper and lower punch forces during the compression in Fig. 1 shows that a substantial difference existed. When

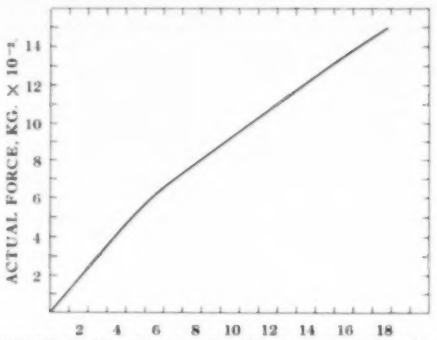


Fig. 5.—Calibration curve for converting upper punch force oscillograph record to force exerted.

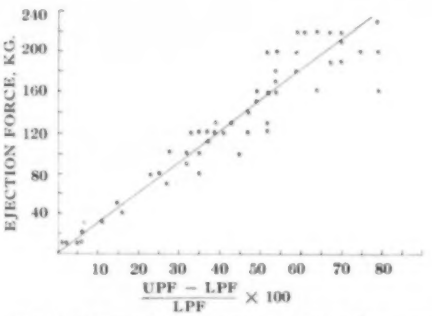


Fig. 6.—The relation between ejection force and the percentage of force exerted by the upper punch taken by the die wall for the compression of a number of tablets from sulfathiazole granulation, both unlubricated and lubricated with various substances. UPF and LPF are, respectively, upper punch force and lower punch force.

the same material was compressed under identical conditions, except for addition of lubricant, the maximum upper punch force was lower and the difference between the upper and lower punch force was reduced significantly as shown in Fig. 2.

As determined from Figs. 1 and 2 and the calibration curve shown in Fig. 5, the maximum forces were as follows:

	Unlubricated, Kg.	Lubricated, Kg.
Upper punch	1390	1010
Lower punch	760	980

There was a similar reduction in ejection forces; i. e., 210 Kg. *vs.* about 20 Kg. for the unlubricated and lubricated granulations, respectively.

As shown in Figs. 3 and 4, in the compression of lactose and sodium iodide there was also a marked difference between the upper and lower punch forces. Because the ejection force was comparatively small, the recording sensitivity of the lower punch was increased as indicated in each of these figures.

The complete results of compressions of lubricated sulfathiazole granulation are given in Table III and IV. In these tables, the value *R* represents

TABLE III.—EFFECT OF VARIOUS SUBSTANCES TESTED AS TABLET LUBRICANTS FOR COMPRESSION OF A SULFATHIAZOLE GRANULATION

Lubricant		Maximum Upper Punch Force, Kg.	Ejection Force, Kg.[a]	R[b]	Lubricant		Maximum Upper Punch Force, Kg.	Ejection Force, Kg.[a]	R[b]
Kind	Amount in %				Kind	Amount in %			
None (Control)	..	1,310	220	0.63	Carbowax® 4000	2	1,170	100	0.74
None (Control)	..	1,255	160	0.66	Carbowax® 4000	2	1,125	90	0.76
None (Control)	..	1,300	220	0.63	Control	..	1,385	230	0.56
Calcium Stearate	1/2	1,030	10	0.95	Control	..	1,365	220	0.59
Calcium Stearate	1/2	1,010	10	0.96	Control	..	1,340	220	0.61
Calcium Stearate	1/2	1,025	10	0.96	Control	..	1,340	220	0.60
Calcium Stearate	1	985	10	0.98	Boric acid	1/2	1,215	80	0.81
Calcium Stearate	1	995	10	0.98	Boric acid	1/2	1,255	100	0.76
Calcium Stearate	1	1,000	10	0.98	Boric acid	1/2	1,230	100	0.75
Calcium Stearate	2	1,015	10	0.98	Boric acid	1	1,230	130	0.72
Calcium Stearate	2	995	10	0.99	Boric acid	1	1,315	160	0.67
Calcium Stearate	2	995	10	0.99	Boric acid	1	1,230	120	0.72
Sodium Stearate	1/2	1,075	50	0.87	Boric acid	2	1,255	130	0.72
Sodium Stearate	1/2	1,080	40	0.86	Boric acid	2	1,200	100	0.78
Sodium Stearate	1/2	1,105	40	0.86	Boric acid	2	1,255	130	0.70
Sodium Stearate	1	1,030	20	0.94	Phenacetin	1/2	1,220	120	0.72
Sodium Stearate	1	1,035	20	0.94	Phenacetin	1/2	1,285	170	0.65
Sodium Stearate	1	1,065	30	0.90	Phenacetin	1/2	1,250	160	0.67
Sodium Stearate	2	1,010	10	0.95	Phenacetin	1	1,185	120	0.74
Sodium Stearate	2	1,000	10	0.95	Phenacetin	1	1,195	120	0.71
Sodium Stearate	2	1,010	20	0.96	Phenacetin	1	1,200	120	0.71
Aspirin	1/2	1,290	200	0.66	Phenacetin	2	1,215	120	0.71
Aspirin	1/2	1,300	200	0.65	Phenacetin	2	1,200	110	0.73
Aspirin	1/2	1,290	180	0.65	Phenacetin	2	1,195	120	0.73
Aspirin	2	1,280	180	0.65	Talc	1/2	1,330	220	0.60
Aspirin	2	1,240	160	0.66	Talc	1/2	1,330	210	0.59
Aspirin	2	1,300	170	0.65	Talc	1/2	1,350	200	0.56
Spermaceti	1/2	1,350	160	0.56	Talc	1	1,330	190	0.59
Spermaceti	1/2	1,270	130	0.66	Talc	1	1,325	190	0.59
Spermaceti	1/2	1,400	160	0.52	Talc	1	1,300	190	0.60
Spermaceti	1	1,300	160	0.61	Talc	2	1,290	200	0.63
Spermaceti	1	1,330	200	0.57	Talc	2	1,225	140	0.68
Spermaceti	1	1,235	120	0.66	Talc	2	1,235	150	0.67
Spermaceti	2	1,220	120	0.66	Magnesium Stearate	1/2	1,125	40	0.82
Spermaceti	2	1,200	120	0.68	Magnesium Stearate	1/2	1,125	30	0.83
Spermaceti	2	1,185	100	0.69	Magnesium Stearate	1/2	1,110	20	0.85
Veegum	1/2	1,330	220	0.62	Magnesium Stearate	1	1,100	20	0.85
Veegum	1/2	1,330	220	0.62	Magnesium Stearate	1	1,090	20	0.87
Veegum	1/2	1,330	220	0.62	Magnesium Stearate	1	1,090	20	0.85
Veegum	1	1,360	250	0.60	Magnesium Stearate	2	1,080	10	0.87
Veegum	1	1,290	160	0.65	Magnesium Stearate	2	1,060	10	0.88
Veegum	1	1,300	180	0.63	Magnesium Stearate	2	1,080	10	0.87
Veegum	2	1,350	280	0.59	Zinc Stearate	1/2	1,080	20	0.87
Veegum	2	1,300	180	0.63	Zinc Stearate	1/2	1,105	20	0.84
Veegum	2	1,360	260	0.57	Zinc Stearate	1/2	1,090	20	0.85
Carbowax® 4000	1/2	1,170	120	0.75	Zinc Stearate	1	1,075	20	0.85
Carbowax® 4000	1/2	1,140	90	0.76	Zinc Stearate	1	1,100	20	0.85
Carbowax® 4000	1/2	1,125	70	0.79	Zinc Stearate	1	1,090	20	0.86
Carbowax® 4000	1	1,220	120	0.71	Zinc Stearate	2	1,090	20	0.86
Carbowax® 4000	1	1,170	80	0.80	Zinc Stearate	2	1,080	20	0.88
Carbowax® 4000	1	1,155	100	0.75	Zinc Stearate	2	1,090	20	0.85
Carbowax® 4000	2	1,155	80	0.74					

[a] Estimated error ± 10 Kg.

[b] $R = \frac{\text{Maximum lower punch force.}}{\text{Maximum upper punch force}}$

the ratio of the maximum lower punch force to the maximum upper punch force, and is the fraction of maximum force exerted by the upper punch transmitted to the lower punch. The efficiency of a lubricant appears to be measured directly by its R values. Generally, with increased lubricant efficiency, the force necessary for ejection also was greatly decreased.

The nature of the relationship between the ejection force and the force lost to the die wall during the final phase of tablet formation is evident in the plot shown in Fig. 6, where the data for the two variables shown in Table III have been used. Within the precision of the measurement it appears that the two are directly and linearly related. This mutual dependency is certainly not unexpected.

It is evident that the data such as are shown in these tables can be used to evaluate the relative efficiencies of several lubricants. From the values given, it is apparent that the commonly employed stearate lubricants are highly efficient. Others which have been suggested such as Carbowax®,

TABLE IV.—EFFECT OF STEARATES USED AS TABLET LUBRICANTS ON THE COMPRESSION OF A SULFATHIAZOLE GRANULATION

Lubricant	%	Maximum Upper Punch Force, Kg.	Ejection Force, Kg.[a]	R^b
Control	..	1,200	167	0.67
Magnesium Stearate	1/2	970	21	0.97
Magnesium Stearate	1/2	980	16	0.98
Magnesium Stearate	1	960	15	0.98
Magnesium Stearate	1	955	15	0.99
Magnesium Stearate	1	960	17	0.99
Magnesium Stearate	1	945	22	0.99
Magnesium Stearate	2	930	13	1.00
Magnesium Stearate	2	925	13	1.00
Magnesium Stearate	2	945	11	0.99
Magnesium Stearate	2	945	13	1.00
Control	..	1,235	200	0.65
Sodium Stearate	1/2	1,000	48	0.91
Sodium Stearate	1/2	995	40	0.91
Sodium Stearate	1/2	995	60	0.88
Sodium Stearate	1/2	980	24	0.95
Sodium Stearate	1	945	32	0.99
Sodium Stearate	1	970	29	0.93
Sodium Stearate	1	980	37	0.89
Sodium Stearate	1	980	29	0.95
Sodium Stearate	2	930	23	0.96
Sodium Stearate	2	930	23	0.97
Sodium Stearate	2	930	21	0.95
Sodium Stearate	2	915	19	0.98
Control	..	1,155	167	0.68
Calcium Stearate	1/2	930	19	0.93
Calcium Stearate	1/2	925	17	0.96
Calcium Stearate	1/2	925	15	0.94
Calcium Stearate	1/2	925	16	0.95
Calcium Stearate	1	900	16	0.97
Calcium Stearate	1	900	13	0.99
Calcium Stearate	1	910	13	0.96
Calcium Stearate	1	915	13	0.95
Calcium Stearate	1	910	13	0.97
Control	..	1,215	180	0.64
Calcium Stearate	2	995	13	0.95
Calcium Stearate	2	985	15	0.95
Calcium Stearate	2	970	15	0.97
Calcium Stearate	2	980	13	0.96
Calcium Stearate	2	980	13	0.96
Control	..	1,200	167	0.67
Zinc Stearate	1/2	995	20	0.95
Zinc Stearate	1/2	965	24	0.94
Zinc Stearate	1/2	945	20	0.98
Zinc Stearate	1/2	970	21	0.93
Control	..	1,155	167	0.68
Zinc Stearate	1	970	23	0.96
Zinc Stearate	1	945	21	0.99
Zinc Stearate	1	955	21	0.99
Zinc Stearate	1	955	20	0.98
Zinc Stearate	1	930	17	0.97
Zinc Stearate	2	965	16	0.97
Zinc Stearate	2	955	16	0.97
Zinc Stearate	2	955	17	0.96
Zinc Stearate	2	965	20	0.97

[a] The recording sensitivity was increased fivefold to permit more accurate determination of the ejection force; estimated error ± 2 Kg.

[b] $R = \frac{\text{Maximum lower punch force}}{\text{Maximum upper punch force}}$.

boric acid, talc, etc., are comparatively poor lubricants. The minimum useful concentrations of lubricants can also be determined from these measurements. In some cases it appears that the optimum lubricant effect was reached at concentrations as low as 0.5 to 1%.

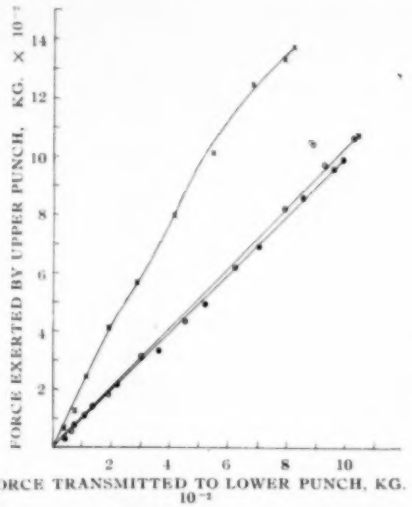


Fig. 7.—The relation between upper and lower punch force for compression of sulfathiazole granulation unlubricated and under the two indicated conditions of lubrication. x, Compression of unlubricated granulation. ○, Die wall only lubricated ●, Granulation lubricated with calcium stearate 2%.

The thickness of all tablets described in Table III was 0.151 inch ± 0.002 inch in spite of substantial differences in the force exerted by the upper punch in their compression. This can be explained by the nature of the relation between compressional force and density which has been previously found to be logarithmic, i. e., the density of a tablet is directly proportional to the logarithm of force (2). This may also be due to the fact that the mean forces for the several tablets did not vary to the same extent as the upper punch force.

The nature of the relationship between upper and lower punch force as well as effect of lubricant on the relation is apparent from Fig. 7. In the case of

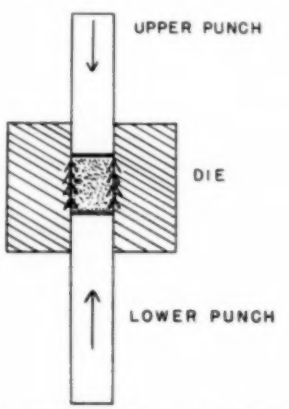


Fig. 8.—A representation of the distribution of forces in the single punch tablet compression process.

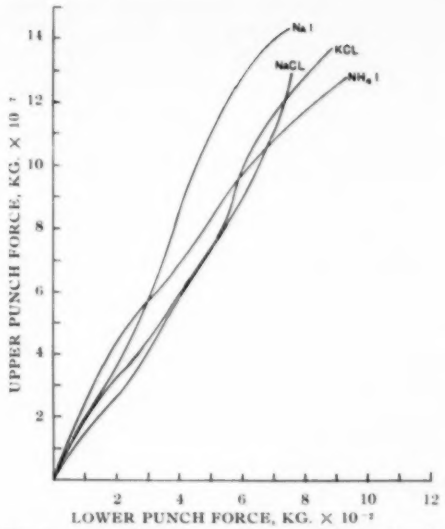


Fig. 9.—Type of upper *vs.* lower punch force relations that existed for several unlubricated salts.

unlubricated granulation the curve has several changes of slope. The slope during any stage of the compression indicates that a significant amount of force exerted by the upper punch did not reach the lower punch. In the cases where either the granulation or the die wall was well lubricated, the slope was close to unity (or 45°), indicating that nearly all the force exerted by the upper punch reached the lower punch. The distribution of forces during single punch compression is illustrated by the drawing in Fig. 8. At all times during compression, the force exerted by the upper punch was distributed between the lower punch and the die wall. Lubricants added to granulation or placed on the wall of the die reduced the vertical die wall force, allowing a larger portion of the exerted force to reach the lower punch.

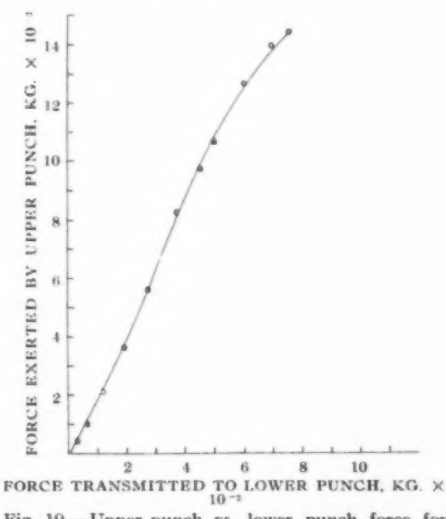


Fig. 10.—Upper punch *vs.* lower punch force for compression of lactose without lubricant added.

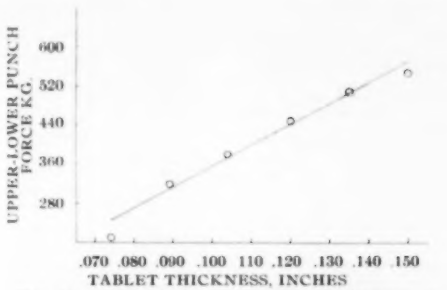


Fig. 11.—The relation between the difference in maximum upper punch and maximum lower punch forces and the thickness of tablets made from varying weights of unlubricated sulfathiazole granulation.

The variation of lower punch force with upper punch force during the compression of the indicated inorganic salts is shown in Fig. 9. Similar data for lactose are presented in Fig. 10. There appears to be no relationship or a typical curve for salts with either a common cation or anion. It should be emphasized here, however that the data for any specific material were reproducible so that the differences are valid and are intrinsic characteristics of the material itself.

In the limited range studied, the force lost to the die wall and the ejection force appear to be linearly dependent on the area of a tablet in contact with the die wall. These relationships are illustrated in Figs. 11 and 12 for the unlubricated sulfathiazole granulation for a range of tablet thickness between 0.075 and 0.150 inch. Data for these graphs were obtained by compressing varying weights of the granulation to the same lower punch force (800 Kg.).

In the case of Fig. 11 the slope of the line represents the force gradient that existed on the surface of the tablet in contact with the die wall at the instant compression was completed for any given final thickness of a tablet in the range of the plot. In this system the gradient was about 9000 lbs.

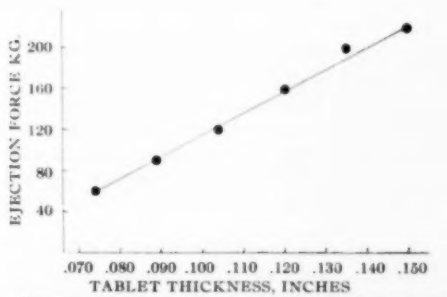


Fig. 12.—The relation between ejection force and tablet thickness for tablets made from compressing varying weights of unlubricated sulfathiazole granulation.

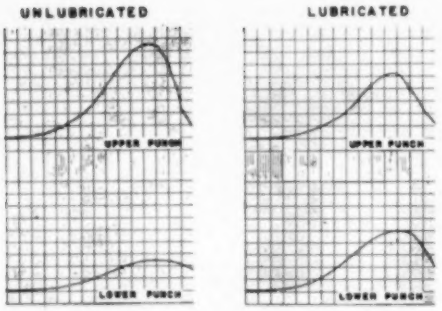


Fig. 13.—The effect of lubricant added to granulation on the transmission of stress in the tablet formed. Compare upper punch curves for the lubricated and unlubricated cases.

(4000 Kg.) per inch. The slope of the line in Fig. 12 represents the force per unit axial length needed to eject the tablet from the die. The value of the gradient here was about 4,600 lbs. (2,100 Kg.) per inch.

The advantage of using concave punches to reduce die wall force losses and to reduce ejection force may be seen from the large gradients that can exist in poorly lubricated systems.

Lubricant added to the granulation apparently played a part in the regularity of transmission of stress through the tablet during the compression. An examination of the upper punch force curves of Fig. 13 for compression of lubricated and unlubricated granulation shows that the curve for the unlubricated granulation is somewhat more irregular than that for the lubricated granulation. This effect is also noticeable for the two lower punch force curves.

CONCLUSIONS AND RECOMMENDATIONS

1. On the basis of the results obtained, it appears possible to evaluate lubricant efficacy, simply and rapidly, by the use of the instrumented tablet machine.

2. It is suggested that a large number of possible lubricants for a given granulation can be quickly screened both as to composition and optimum concentration.

REFERENCES

(1) Higuchi, T., Nelson, E., and Busse, L. W., THIS JOURNAL, **43**, 344(1954).
(2) Higuchi, T., Rao, A. N., Busse, L. W., and Swintosky, J. V., *ibid.*, **42**, 194(1953).

Amides of 5-Nitro-2-Thiophenecarboxylic Acid*

By WILLIAM O. FOYE and JOHN J. HEFFERREN†

A number of new aliphatic, aromatic, and heterocyclic substituted amides of 5-nitro-2-thiophenecarboxylic acid have been prepared for testing as antiviral agents. These compounds are related to nitrobenzene, nitrothiophene, and nitrofuran derivatives which have previously been shown to have antiviral activity.

SINCE the observation that certain nitroacridines showed antiviral activity while other closely related nonnitro acridines had little or no activity (1), the antiviral activity of many simpler aromatic nitro compounds has been investigated. Simple amides of *p*-nitrobenzoic acid and various derivatives of 5-nitro-2-furaldehyde, for instance, have been shown by Eaton (2) to possess inhibitory activity against pneumotropic viruses both in chick embryos and in mice. Hamilton (3) has, moreover, provided evidence that *p*-nitrobenzoic acid derivatives do not owe their activity, against psittacosis virus at least, to the reduced amino compounds. Among the nitro compounds which exhibit antiviral activity, the only chemical characteristic in common seems to be the presence of a nitro group conjugated with an aromatic ring.

More recent research has been concerned with nitroaldehyde derivatives, particularly those with

* Received March 5, 1954, from the School of Pharmacy, University of Wisconsin, Madison.
Abstracted from a dissertation presented to the Graduate School of the University of Wisconsin by John J. Hefferren in partial fulfillment of the requirements for the degree of Doctor of Philosophy, September, 1953.
† Fellow of the American Foundation for Pharmaceutical Education, 1950–1953. Present address: American Medical Association Laboratories, Chicago, Ill.

furan and thiophene rings (4). That nitrothiophene acid amides might have antiviral activity has not yet been determined, although 5-nitro-2-thiophenecarboxamide is highly active against *Streptococcus hemolyticus* (5). The purpose of this research is the preparation of a series of 5-nitro-2-thiophenecarboxamides of relatively low molecular weight for antiviral testing.

These amides were all prepared by the reaction of 5-nitro-2-thiophenecarbonyl chloride with various aliphatic, aromatic, and heterocyclic amines. Reactions were also carried out with ureas, urethan, and an amino acid ester. The methods of preparation used may be described as four general types. (*a*). The more reactive amines formed amides with 5-nitro-2-thiophenecarbonyl chloride at room temperature using two equivalents of a monoamine or one equivalent of a diamine. (*b*) Amide formation with an amino acid involved competition with diketopiperazine formation, which was minimized by reacting the amino acid ester hydrochloride in two equivalents of pyridine at 50°. (*c*) The slightly basic amines required refluxing of the benzene-pyridine suspensions for extended periods. Where diamines were used, it was found advantageous to neutralize the products to avoid mixtures of amide hydrochlorides and free amides which were difficult to separate. (*d*) The formation of ureides involved prolonged refluxing in benzene with an acid catalyst.

The reaction of 5-nitro-2-thiophenecarbonyl chloride with urethan was attempted under a variety of conditions. The method of Ben-Ishai and Katchalski (6), in which a urethan is heated with acetyl chloride, was unsuccessful because 5-nitro-2-thiophenecarbonyl chloride is a high-melting solid and urethan decomposes above 50°. Allowing the reactants to remain in pyridine-ether solution for a week at room temperature gave no reaction, nor did refluxing in benzene with excess urethan for extended periods.

The 5-nitro-2-thiophenecarboxamides, which are described in Table I, include examples with aliphatic, cycloaliphatic, aromatic, and heterocyclic substituents on the amide nitrogen. Amino components were selected with as low a molecular weight as permissible to include the types mentioned and maintain some degree of water solubility. Water-solubilizing groups were included in the case of the amides prepared from β-diethylaminoethylamine and ethyl glycine. The ureide and thioureide derivatives were prepared to provide an analogy to the semicarbazone and thiosemicarbazone derivatives of the nitrofuran and nitrothiophene aldehydes already shown to have some antiviral activity.

A majority of these amides, as well as the unsubstituted 5-nitro-2-thiophenecarboxamide, are being tested at the Lilly Research Laboratories for antiviral activity. The results obtained to date on several of the more water-soluble derivatives, the allyl, ethyl glycinyl, and pyrrolidyl, show them to be ineffective against influenza and encephalitis type viruses.[1]

EXPERIMENTAL

2-Iodothiophene.—The method of Minnis (7) was used, and 50–60% yields of colorless liquid were obtained; b. p. 46–52° (2 mm.).

5-Nitro-2-iodothiophene.—This compound was prepared in 60–65% yields by the nitration of 2-iodothiophene with concentrated nitric acid in acetic anhydride according to the procedure of Dann (8); m. p. 73–74°.

5-Nitro-2-thiophenecarboxylic Acid.—The 5-nitro-2-iodothiophene was converted to 5-nitro-2-cyanothiophene by treatment with cuprous cyanide and pyridine according to the method of Dann (8). The nitrile was not isolated but hydrolyzed immediately with concentrated hydrochloric acid to give 40–53% yields of 5-nitro-2-thiophenecarboxylic acid; m. p. 155–156°.

5-Nitro-2-thiophenecarbonyl Chloride.—A mixture of 8.6 Gm. (0.05 mole) of 5-nitro-2-thiophenecarboxylic acid and 30.0 Gm. (0.25 mole) of thionyl chloride was refluxed on a steam bath for one hour. The yellow solution was poured into a large evaporating dish and the excess thionyl chloride was removed by blowing dry air across the surface. The yellow 5-nitro-2-thiophenecarbonyl chloride was then taken up in 50 cc. of dry benzene. The solution was cooled in an ice bath for several minutes and used immediately for the preparation of the amides.

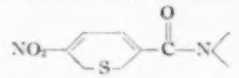
5-Nitro-2-thiophenecarboxamides—*Procedure A.*—This procedure can be illustrated by the reaction with allylamine. To the slightly cooled benzene solution of 5-nitro-2-thiophenecarbonyl chloride in a 250-cc. Erlenmeyer flask was added dropwise and with shaking or stirring 5.7 Gm. (0.10 mole) of allylamine in 10 cc. of dry benzene. An immediate reaction took place with considerable evolution of heat and the formation of a voluminous precipitate. The reaction was allowed to proceed at room temperature for three hours with occasional shaking.

The reaction mixture was diluted with 50–100 cc. of chloroform which dissolved the precipitate. The benzene-chloroform solution was thoroughly extracted with water to remove allylamine hydrochloride and unreacted allylamine, and was then dried over anhydrous sodium sulfate. After removal of the solvents, the residue was recrystallized from aqueous alcohol, and 9.6 Gm. (85%) of N-allyl-5-nitro-2-thiophenecarboxamide was obtained as fine, white needles which melted at 113–114°.

In the preparations of N-(β-diethylaminoethyl)-5-nitro-2-thiophenecarboxamide hydrochloride and N-ethyl-N′-(5-nitro-2-thiophenecarbonyl)-piperazine hydrochloride, no pyridine was used and the original precipitate was merely washed with anhydrous ether before recrystallization.

[1] The authors are indebted to K. K. Chen and W. S. Boniece of the Lilly Research Laboratories for these results.

TABLE I.—5-NITRO-2-THIOPHENECARBOXAMIDES



No.[a]	Amine Component	Method	Crystallized from:	M. P.,[b] °C.	Yield, %	Formula		Analyses, % Calcd.	Found[c]
1	Allylamine	A	Ethanol-water	113–114	85	$C_8H_8O_3N_2S$	C:	45.27	45.58
							H:	3.90	4.02
2	β-Diethylamino-ethylamine[d]	A	Abs. ethanol	200–202	60	$C_{11}H_{18}O_3N_3ClS$	C:	42.92	42.10
							H:	5.89	5.83
3	Ethyl glycine--HCl	B	Ethanol-water	122–124	42	$C_9H_{10}O_5N_2S$	C:	41.85	42.11
							H:	3.90	3.81
4	Piperidine	A	Ethanol-water	94–95	75	$C_{10}H_{12}O_3N_2S$	C:	49.98	50.16
							H:	5.03	5.07
5	Morpholine	A	Ethanol-water	89–90	80	$C_9H_{10}O_4N_2S$	C:	44.62	44.95
							H:	4.16	4.36
6	2-Aminothiazole	C	Acetic acid	>330	80	$C_8H_5O_3N_3S_2$	C:	37.64	38.52
							H:	1.97	2.17
7	2-Aminopyridine	C	Ethanol	198–200	65	$C_{10}H_7O_3N_3S$	C:	48.19	48.19
							H:	2.83	2.65
8	N-Ethylpiper-azine[d,e]	A	Abs. methanol-ether	262–264[d]	56	$C_{11}H_{16}O_3N_3ClS$	C:	43.20	43.46
							H:	5.28	5.39
9	Pyrrolidine	A	Ethanol-water	179–180	75	$C_9H_{10}O_3N_2S$	C:	47.77	48.10
							H:	4.56	4.78
10	*m*-Bromoaniline	C	Ethanol	232–234	89	$C_{11}H_7O_3N_2BrS$	C:	40.50	40.93
							H:	2.16	2.10
11	*m*-Nitroaniline	C	Acetic acid	204–205	80	$C_{11}H_7O_5N_3S$	C:	45.05	45.07
							H:	2.41	2.85
12	*p*-Nitroaniline	C	Acetic acid	274–275	90	$C_{11}H_7O_5N_3S$	C:	45.05	45.35
							H:	2.41	2.65
13	Benzylamine	A	Ethanol-water	117–118	95	$C_{12}H_{10}O_3N_2S$	C:	54.95	55.63
							H:	3.84	4.02
14	Urea	D	Acetic acid	216–218	67	$C_6H_5O_4N_3S$	C:	33.49	32.85
							H:	2.34	2.39
15	Thiourea	D	Ethanol	205–206	64	$C_6H_5O_3N_3S_2$	C:	31.16	31.35
							H:	2.18	2.17

[a] The colors of these amides were various shades of yellow in general; compounds 1, 3, 8, 13, and 14 were white, and compounds 2 and 15 were tan.
[b] The melting points are corrected.
[c] The analyses were carried out by the Clark Microanalytical Laboratory, Urbana, Ill.
[d] The hydrochlorides were isolated.
[e] The N-ethylpiperazine was prepared by the procedure of Moore, T. S., Boyle, M., and Thorn, V. M., *J. Chem. Soc.*, **1929**, 46; b. p. 152–155°.

Procedure B.—A solution of 5-nitro-2-thiophenecarbonyl chloride, from 4.2 Gm. (0.025 mole) of the corresponding acid, in 100 cc. of dry benzene was placed in a 500-cc. flask equipped with a mercury-sealed stirrer and reflux condenser. After the addition of 3.5 Gm. (0.025 mole) of ethylglycine hydrochloride (9) and 3.5 Gm. (0.05 mole) of redistilled pyridine (dried over potassium hydroxide), the stirred mixture was allowed to react at room temperature for thirty minutes. It was then warmed at 50° for another thirty minutes, and finally stirred at room temperature for two hours.

The deep red benzene solution was diluted to twice its volume with Skellysolve® B. The precipitate obtained on cooling was recrystallized from aqueous alcohol, and 2.7 Gm. (42%) of ethyl N-(5-nitro-2-thiophenecarbonyl)-glycine was obtained which melted at 122–124.°

Procedure C.—Amide formation with 2-aminothiazole is a typical example of this method. A solution of 5-nitro-2-thiophenecarbonyl chloride, from 4.5 Gm. (0.026 mole) of the corresponding acid, in 50 cc. of dry benzene was placed in a 500-cc. flask equipped with a mercury-sealed stirrer and reflux condenser. After the addition of 2.6 Gm. (0.026 mole) of 2-aminothiazole and 2.1 Gm. (0.026 mole) of redistilled pyridine in 150 cc. of dry benzene, the suspension was refluxed for three hours and then stirred at room temperature for three hours.

In the case of 2-aminopyridine, the mixture was refluxed for twelve hours. *m*-Bromoaniline required about two hours of heating, while the nitroanilines required about ten hours.

During the heating, a flocculent, yellow precipitate gradually formed. The precipitate was filtered and washed with several portions of water, 5% sodium carbonate solution, and dilute hydrochloric acid to remove pyridine hydrochloride, unreacted 2-aminothiazole, and to neutralize any amide hydrochloride. The residue was then recrystallized from glacial acetic acid, and 5.3 Gm. (80%) of N-(2′-thiazolyl)-5-nitro-2-thiophenecarboxamide was obtained in the form of micro crystals which did not melt below 330°.

Procedure D.—The formation of the 5-nitro-2-thiophenecarbonyl ureide is representative of this procedure. A solution of 5-nitro-2-thiophenecarbonyl chloride, from 4.5 Gm. (0.026 mole) of 5-nitro-2-thiophenecarboxylic acid, in 40 cc. of dry benzene was placed in a 250-cc. flask fitted with a mercury-sealed stirrer and reflux condenser. To this solution was added 1.6 Gm. (0.026 mole) of urea suspended in 100 cc. of dry benzene and one drop of concentrated sulfuric acid. The suspension was

stirred at reflux temperature for six hours and at room temperature overnight.

During the refluxing a light gray precipitate gradually accumulated. The precipitate was removed and thoroughly washed with Skellysolve® B. After recrystallization from glacial acetic acid, 3.8 Gm. (67%) of 5-nitro-2-thiophenecarbonyl ureide was collected, m. p. 216–218°.

SUMMARY

1. General procedures for preparing aliphatic, aromatic, and heterocyclic substituted amides of 5-nitro-2-thiophenecarboxylic acid are presented. These compounds were prepared for antiviral testing.

2. The following new N-substituted amides of 5-nitro-2-thiophenecarboxylic acid have been characterized: allyl, β-diethylaminoethyl hydrochloride, ethyl glycinyl, piperidyl, morpholyl, 2′-thiazolyl, 2′-pyridyl, N′-ethylpiperazinyl hydrochloride, pyrrolidyl, *m*-bromophenyl, *m*-nitrophenyl, *p*-nitrophenyl, benzyl, and the ureide and thioureide.

REFERENCES

(1) Eaton, M. D., Van Allen, A., and Wiener, A., *Proc. Soc. Exptl. Biol. Med.*, **66**, 141(1947); Eaton, M. D., *Ann. Rev. Microbiol.*, **4**, 223(1950).
(2) Eaton, M. D., Huang, C.-T., and Levenson, C. G., *Proc. Soc. Exptl. Biol. Med.*, **71**, 501(1949).
(3) Hamilton, H. L., *ibid.*, **59**, 220(1945).
(4) Eaton, M. D., Perry, M. E., and Gocke, I. M., *ibid.*, **77**, 422(1951); Minton, S. A., Officer, J. E., and Thompson, R. L., *J. Immunol.*, **70**, 222(1953).
(5) Johnson, O. H., Green, D. E., and Pauli, R., *J. Biol. Chem.*, **153**, 37(1944).
(6) Ben-Ishai, D., and Katchalski, E., *J. Org. Chem.*, **16**, 1025(1951).
(7) Minnis, W., "Organic Syntheses," Coll. Vol. II, John Wiley & Sons, Inc., New York, 1943, p. 357.
(8) Dann, O., *Ber.*, **76**, 425(1943).
(9) Curtius, T., and Koch, F., *J. prakt. Chem.*, **146**, 487 (1888).

Ultraviolet Irradiation and Absorptiometric Methods for the Determination of Diethylstilbestrol in Tablets*

By JOHN M. GOODYEAR, L. SHIRL HATFIELD, and MAX M. MARSH

The present official method for the determination of diethylstilbestrol is not specific, and incomplete extraction of active material from small dosage tablets is often encountered. To circumvent these difficulties, two new methods of assay have been developed. The procedures depend on photochemical changes which diethylstilbestrol undergoes when it is irradiated with short wavelength ultraviolet light and on the reproducible shift of the ultraviolet absorption spectrum of diethylstilbestrol in acid and in alkaline solution. Apparatus for the irradiation method includes a suitable strong ultraviolet light source and a spectrophotometer capable of measuring visible light absorption, while the ultraviolet absorptiometric method requires a spectrophotometer operating in the ultraviolet region. Interferences in the irradiation procedure are primarily inhibitory in nature while those in the UV absorptiometric method are those compounds which exhibit a shift in absorption spectrum in the 280 mμ range in acid and alkaline solution. The nature of the pigment produced by irradiation is not known.

Numerous colorimetric, spectrophotometric, and chemical methods for the determination of diethylstilbestrol have been described in recent years (1–9, 11). These derive their operational utility from quantitative reactivity with the phenol moiety of the compound. In the case of the present official method (10), other reducing substances which are not necessarily phenols will produce positive interferences.

In attempting to apply the extraction and color development procedures which are official in the U. S. P. XIV to tablets of diethylstilbestrol, some difficulties have been encountered in obtaining quantitative recoveries of active ingredients, particularly in the case of low-concentration forms, i. e., 0.1- and 0.25-mg. tablets.

Utilizing samples of pure diethylstilbestrol (U. S. P. reference standard), it has been demonstrated (Table I) that an approximate 3 per cent loss of active material consistently occurs during

* Received March 10, 1954, from Eli Lilly and Co., Indianapolis 6, Ind.

the extraction steps. In an attempt to overcome these losses, alternate methods of extraction and color development were investigated.

TABLE I.—STANDARD RECOVERY

	Method	Recovery, %	Remarks
A	Irradiation	98.3 100 98.5 99.6	0.10 mg. of standard added to a blank granulation equivalent to 0.10-mg. tablet
B	Ultraviolet absorption	99.7 100.6 98.9 98.6	0.25 mg. of standard added to a blank granulation equivalent to 0.25-mg. tablet
C	United States Pharmacopeia	96.3 97.5 96.6 97.5	U. S. P. reference standard

The present U. S. P. (XIV) method for the determination of diethylstilbestrol in pharmaceutical preparations utilizes the reducing power of phenolic structure of the compound to produce a "heteropoly blue" which is not particularly specific. In our experience, small but appreciable amounts of positive interference are consistently encountered in assaying low-dosage tablet preparations by initial methanol extraction of the tablet mixtures.

An observation made by one of us (J. M. G.) during the course of some deterioration studies on diethylstilbestrol indicated that on exposure to intense ultraviolet light of short (250 mμ) wavelength, solutions of the compound developed a yellow color. Studies on the absorption of ultraviolet light by diethylstilbestrol in solutions of varying *p*H indicated a pronounced absorbancy difference at wavelengths sufficiently long (280 mμ) to escape much of the interference encountered in extracts from tablet preparations (M. M.). These two observations are made the basis, respectively, of the two methods of assay subsequently described in this paper.

EXPERIMENTAL A. IRRADIATION METHOD

Reagents.—(*a*) A stock solution of U. S. Pharmacopeia reference diethylstilbestrol containing 0.50 mg. per cc. of glacial acetic acid. This solution should be stored in the refrigerator in a brown glass bottle.

(*b*) Dilutions are prepared by adding 10 cc. of distilled water to each volumetric flask and 1 cc., 2 cc., and 3 cc. of the diethylstilbestrol stock solution to each respective flask. Sufficient glacial acetic acid is added to make 50 cc. These constitute the working standards.

Apparatus.—(*a*) Hanovia Examalite with Burner No. L247 without filter.

(*b*) Beckman DU Spectrophotometer or comparable instrument with matched 1-cm. absorption cells.

(*c*) At least four transparent quartz tubes for irradiation of sample. Tubes should be checked for match by using the standard in all tubes. These may be purchased from H. S. Martin Co., 1916 Greenleaf Street, Evanston, Ill.

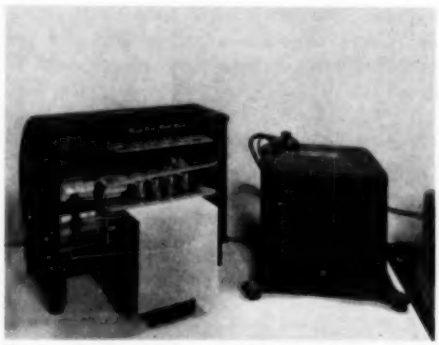


Fig. 1.—Equipment for the short wavelength ultraviolet irradiation.

Preparation of Samples.—No special treatment is required. An amount of a suitably ground sample equivalent to 1.0 mg. of diethylstilbestrol is transferred to a 50-cc. flask and digested with 10 cc. of water with the aid of heat for fifteen minutes. Exposure to high temperatures for extended periods of time should be avoided. The sample is cooled and sufficient glacial acetic acid is added to make 50 cc.; the flask is inverted several times to insure adequate mixing. Note that all standards and sample solutions are made up to contain 20% water; the remainder of the solvent is acetic acid. The sample is filtered or the suspended material allowed to settle out.

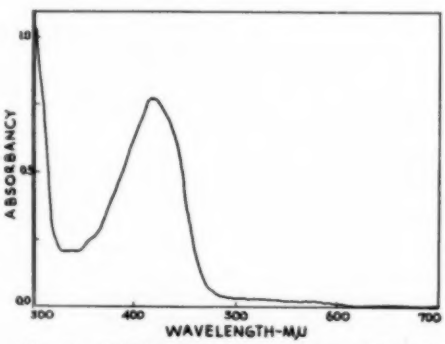


Fig. 2.—Visible absorption spectrum of the pigment produced by irradiation of 40 μg. of diethylstilbestrol per cc. in glacial acetic acid:water. Exposure time seven minutes at a distance of 6 inches from mercury arc lamp.

Procedure.—A suitable amount of solution is transferred to a quartz tube. The quantity is not critical for irradiation but no obstruction such as a cross section of a tube rack should be in front of the sample. The tubes are placed on a suitable stand so the sample within the tube is perpendicular to the light source. The distance from the lamp is 6 inches; this distance should not be changed during an irradiation. In order to avoid variations due to variable thickness of the quartz tubes, they must be placed each time in the same position in front of the lamp. Figure 1 demonstrates the essential components of the irradiation apparatus which can simultaneously irradiate four tubes. After seven minutes' exposure to the full intensity of the lamp, the tubes are immediately removed from the apparatus and cooled. Absorbance is read at 420 mμ using a suitable spectrophotometer or colorimeter and distilled water as a reference solvent. The diethylstilbestrol content is estimated by dividing the standard absorbance into the unknown and multiplying by dilution factors and standard concentration. A blank on tablet granulations without diethylstilbestrol amounts to less than 1% of the total absorbance, while the reagents produce no absorbance at the maximum of 420 mμ. A standard is irradiated each time with the unknown in order to compensate for variation of lamp intensity. The absorbance at 420 mμ for a 20 μg./cc. sample is approximately 0.480 with our equipment and 1-cm. absorption cells.

EXPERIMENTAL B. ULTRAVIOLET DIFFERENCE METHOD

Reagents.—(*a*), 0.5 *N* HCl, (*b*) 0.5 *N* NaOH, (*c*) absolute methanol, reagent grade.

Apparatus.—(*a*) Beckman Model DU spectrophotometer or comparable instrument equipped for measurement of the absorption of ultraviolet light.

(*b*) Matched 1-cm. silica absorption cells.

Procedure.—Transfer a *finely ground* sample equivalent to 2.5 mg. diethylstilbestrol to a dry 50-cc. volumetric flask with the aid of 10 cc. of absolute methanol. With the flask orifice partially closed by insertion of a small funnel, the mixture is boiled *gently* on a steam bath for thirty minutes. The sample is diluted to the mark with absolute methanol, mixed well, and filtered through Whatman No. 1 filter paper, discarding the first 10 cc. of filtrate. Five cubic centimeter aliquots of the filtrate are transferred to each of two 25-cc. volumetric flasks; one of these contains 5 cc. of 0.5 *N* HCl, and the other, 5 cc. of 0.5 *N* NaOH. The solutions are then diluted to about 20 cc. with absolute methanol, mixed well, and diluted to the mark with the same solvent. After mixing, the absorbancy of the samples is measured at 280 mμ against a reference solution of distilled water. Simultaneously, reagent blanks consisting of 5 cc. of 0.5 *N* HCl and 5 cc. 0.5 *N* NaOH, each diluted to 25 cc. with absolute methanol, are prepared.

An aliquot of U. S. P. reference standard diethylstilbestrol in absolute methanol equivalent to 0.25 mg. of diethylstilbestrol is transferred to each of two 25-cc. volumetric flasks, one of which contains 5 cc. of 0.5 *N* HCl and the other, 5 cc. of 0.5 *N* NaOH. The standard is subjected to the same subsequent procedure as the sample.

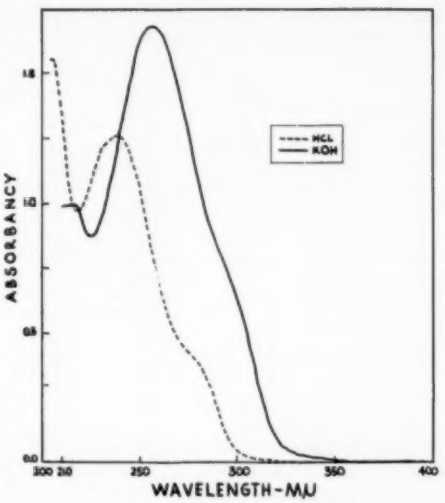


Fig. 3.—Absorption curves of diethylstilbestrol in 0.5 *N* alkali and 0.5 *N* HCl (aqueous alcoholic solution). Concentration: 20 μg./cc.

Calculations.—(*a*) All readings are corrected for the reagent blank as follows:

$$\begin{aligned} &\text{Sample } A_{acid} - \text{blank } A_{acid} = A_{xa} \\ &\text{Sample } A_{base} - \text{blank } A_{base} = A_{xb} \\ &\text{Standard } A_{acid} - \text{blank } A_{acid} = A_{sa} \\ &\text{Standard } A_{base} - \text{blank } A_{base} = A_{sb} \end{aligned}$$

(*b*)
$$\frac{A_{xb} - A_{xa}}{A_{sb} - A_{sa}} = \frac{A_x}{A_s}$$

(*c*)
$$\frac{A_x}{A_s} \times \text{theoretical concentration per tablet} = \text{mg. diethylstilbestrol per tablet}$$

DISCUSSION

It has been demonstrated that both the irradiation and absorbancy difference procedures obey the Bouger-Beer law in the concentration ranges indicated in the *Experimental* section. The absorption curves in Figs. 2 and 3 indicate the visible absorption spectrum for the irradiated solutions and the ultraviolet absorption spectra of acid and alkaline solutions of diethylstilbestrol.

Some studies on the effect of variables in each method have been made. Thus it has been shown that with the conditions described, the visible color intensity developed on irradiation of tablet extracts reaches a maximum in seven minutes. While the simultaneous irradiation of a standard eliminates the time of exposure to the short wavelength ultraviolet light as a variable, maximum sensitivity is achieved by adhering closely to this exposure time. Other solvent combinations such as toluene:acetic acid [96:4 (v/v)], chloroform, or glacial acetic acid require different exposure times for maximum intensity; none of these is as effective an extractant for tablet preparations as the 80:20 acetic acid:water mixture.

In the absorbancy difference procedure, the effect of the final concentrations of acid and of water in

TABLE II.—ASSAY OF ONE LOT OF TABLETS DIETHYLSTILBESTROL LABELED 0.25 MG.

Sample	U. V. Irradiation, mg.	U. V. Absorption Difference, mg.	U. S. P., mg.
1	0.251	0.229	0.236
2	0.254	0.232	0.231
3	0.249	0.238	0.239
4	0.247	0.234	0.239
5	0.252	0.233	0.241
6	0.251	0.230	0.229
7	0.251	0.226	0.237
8	0.264[a]	0.226	0.241
9	...	0.234	0.235
10	...	0.232	...
Average	0.251	0.231	0.236
Standard deviation	0.00234	0.00377	0.00417
Coefficient of variation, %	0.94	1.51	1.67

[a] Since the variation of this value from the mean was more than twice the standard deviation, it was eliminated from the final calculation of standard deviation and coefficient of variation.

the solutions to be examined has been shown to be negligible over a fairly wide range (two-fold increase or decrease). The effect of variation of alkali concentration is somewhat more pronounced but is still not critical. The introduction of increasing amounts of positive interference from the extraction of tablet excipients with methanol-containing increasing amounts of water (10%, 20%, etc.) has been demonstrated; thus one essential feature of the methanol extraction process is the necessity for keeping the water content of the extracting medium at a minimum.

Certain specific interferences aside from those already described have been encountered in both methods. Inhibitory effects in the irradiation procedure have been observed when such substances as vegetable oils, chlorides, sulfates, alcohols, and methyl testosterone are present. The ultraviolet absorption difference method will encounter positive interferences from the presence of other substances capable of forming a phenolate ion in alkaline solution. In some cases, the amount of interference which occurs from other active components may be calculated from their assay obtained by other means and this known interference can be subtracted from the observed absorbancy differences. Such a procedure has been utilized in measuring the amount of diethylstilbestrol in tablets containing this compound in combination with methyl testosterone.

Investigations as to the nature of the photochemical reaction which occurs when solutions of diethylstilbestrol are irradiated with ultraviolet light have not been particularly successful. Reproduction of this reaction by chemical means has not been achieved. Several oxidants have been shown to react with the molecule, but none have produced the characteristic yellow pigment; thus quinone formation seems doubtful.

An investigation of the relative precision of the two methods as compared to the present U. S. P. XIV procedure has yielded the data reported in Table II.

Several production lots of tablets of diethylstilbestrol have been examined and the comparative results reported in Table III. In this connection, it should be noted that the absolute methanol extraction required in the absorbancy difference procedure occasionally fails to remove the active ingredient completely from the tablet excipients; this is particularly true of the 0.1-mg. dosage forms. The inhibitory effect of methyl testosterone in the irradiation procedure may also be observed in tablets containing this compound in combination with diethylstilbestrol.

TABLE III.—COMPARATIVE ASSAYS

Sample	Per Cent of Label Claim: U. S. P.	Irradiation	Ultraviolet Absorption	Tablet, Size, mg.
1	97.3	98.9	110.1	0.1
2	96.4	97.6	104.0	1
3	94.1	94.2	94.1	5
4	95.5	96.3	101.6	5
5	95.8	98.7	97.2	5
6	99.3	100.6	98.0	1
7	99.0	97.0	100.2	1
8	88.6	101.7	68.6	0.1
9	101.2	97.7	92.5	0.5
10	86.7–94.3 (5 assays)	55.7	95.0	0.25[a]

[a] Tablets also contain 5 mg. methyl testosterone.

SUMMARY

Two methods for the determination of diethylstilbestrol in tablet preparations are described. The first procedure utilizes the development of a yellow pigment on irradiation of an aqueous acetic acid solution of the compound by ultraviolet light as a measure of concentration; the second method relates absorbancy differences of acid and alkaline solutions of diethylstilbestrol in the ultraviolet range to concentration.

Results of studies of variables in the procedures are reported together with some limited observations concerning interferences encountered with each method.

Precisions of the methods have been shown to be about ±1% for the irradiation procedure and about ±1.5% for the absorbancy difference procedure.

REFERENCES

(1) Dechene, E. B., THIS JOURNAL, **30**, 208(1941).
(2) Dingemanse, E., *Nature*, **145**, 825(1940).
(3) Elvidge, W. F., *Quart. J. Pharm. and Pharmacol.*, **12**, 347(1939).
(4) Gottlieb, S., THIS JOURNAL, **37**, 147(1948).
(5) Kelly, C. A., and James, A. R., *ibid.*, **41**, 97(1952).
(6) Malpress, F. H., *Biochem. J.*, **39**, 95(1945).
(7) Sondern, C. W., and Burson, C., *Ind. Eng. Chem., Anal. Ed.*, **14**, 358(1942).
(8) Teague, R. S., and Brown, A. E., *J. Biol. Chem.*, **189**, 343(1951).
(9) Tubis, M., and Bloom, A., *Ind. Eng. Chem., Anal. Ed.*, **14**, 309(1942).
(10) "United States Pharmacopoeia," 14th rev., Mack Publishing Co., Easton, Pa., p. 174.
(11) Warren, F. L., Goulden, F., and Robinson, A. M., *Biochem. J.*, **42**, 151(1948).

The Coloring of the Mint Spirits*

By ROBERT J. DONOVAN,† LESLIE M. OHMART,‡ and MITCHELL J. STOKLOSA‡

The colors of three series of samples prepared with certain coal tar dyes were investigated. The colors of the samples were compared by the tristimulus method before and after storage for six weeks in containers of various types. Results of the study indicate that the color of the mint spirits is variable and unstable. Preliminary aqueous maceration of the mint leaves in two separate portions of water gives a color that is slightly more stable than that obtained by the single aqueous maceration of the official method. Of the three series of samples that were prepared with coal tar dyes, only those prepared with Fast Green FCF showed greater color stability than the official products, but the color of these samples was not a close match to that of the official spirits.

Among the official spirits there are two mint spirits, peppermint spirit, U. S. P., and spearmint spirit, N. F., which are different from the others by having a distinctive green color. However, the method now used to color them results in a color that is liable to vary from lot to lot of the spirits because of the use of an unstandardized product. Furthermore, this method is time consuming and gives a color of rather poor stability.

In view of these factors, a study of the colors obtained by the use of some of the certified coal tar dyes, in comparison with the color obtained in the spirits by the use of the official process, was undertaken. Since comparisons were being made with spirits prepared by the official method, it was also decided to check the color stability of spirits prepared by the official method and by slight modifications of it. To simplify the comparisons, and because of its more widespread use, peppermint spirit was used as the type spirit in the experimental work.

When alcoholic dilutions of the mint oils were first adopted in the Fourth Revision of the U. S. P. under the class of spirits, the color was obtained by a single alcoholic extraction of the color in the leaves. Following the suggestions of Utech (1) and Williams (2), the Ninth Revision of the U. S. P. adopted the use of a preliminary maceration of the leaves in water to remove the undesirable reddish color in the leaves, followed by extraction of the expressed marc with alcohol. This method has been retained up till now. However, Griffith and Tice have suggested the use of chlorophyll or of a suitable certified dye as the coloring agent (3), and Ohmart has reported that one or two hours of alcoholic maceration was sufficient to give a satisfactory depth of color (4)

* Received August 21, 1953, from the Massachusetts College of Pharmacy, Boston.
Presented to the Scientific Section, A. Ph. A., Salt Lake City meeting, August, 1953.

† Abstracted from a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by Robert J. Donovan in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

‡ Department of Pharmacy, Massachusetts College of Pharmacy, Boston.

EXPERIMENTAL

The work performed in this investigation consisted essentially of measurements of the colors obtained in solutions by the use of a series of different coloring agents followed by a comparison of each color with the others and with itself to see if and how it changed with the passage of time. The standard color, which was to be duplicated if possible, was that obtained by the official method; but this in itself could not serve as a standard because this color may vary and may not be stable. In order to obtain a standard which would be independent of variation in the colors of the official spirits and also independent of any errors caused by the visual acuity of the observer, recourse was had to the tristimulus method of color analysis and specification.[1]

The instrument used to obtain the spectral transmissions was a Beckman Model DU quartz spectrophotometer. The light path in the solution was 1.000 cm. The solution blank used was a 10% (v/v) solution of peppermint oil in alcohol. The sensitivity control was set to obtain maximum sensitivity. A nominal slit width setting of 0.02 mm. was used in order to obtain uniformity of comparison in different regions of the spectrum from sample to sample. Readings were made at 10 mμ intervals in the region from 380 to 770 mμ, inclusive. Since the procedure adopted did not require absolute values, but only involved comparisons among different samples before and after storage, no attempt was made to calibrate the instruments so as to give absolute tristimulus values. Values for the weighting factors used were those for Standard Illuminant A (gas-filled incandescent lamps) and the Standard Observer of the International Commission on Illumination.

For a check on the stability of the colors, the spirits were exposed to north daylight for a period of six weeks while stored in flint glass, amber glass, green glass, and in flint glass in lightproof outer containers, to determine whether there was any variation in stability that resulted from the type of container used.

Four samples were prepared by the official

[1] For a full discussion of this and other methods of color analysis *cf.* Deane B. Judd, "Measurement and Specification of Color," in *Analytical Absorption Spectroscopy*, Ed. M. G. Mellon, John Wiley & Sons, Inc., New York, 1950, Ch. 9, pp. 515–600.

TABLE I.—TRISTIMULUS VALUES OF SAMPLES COLORED WITH MINT LEAVES

Sample	Initial Values			Values after Storage in Flint Glass		
	x	y	Y/Y_0	x	y	Y/Y_0
1	0.5072	0.4684	0.3254	0.4745	0.4263	0.8468
2	0.4562	0.5175	0.4297	0.4876	0.4807	0.5603
3	0.4526	0.5204	0.4256	0.4968	0.4628	0.6556
4	0.4518	0.5220	0.4112	0.4855	0.4332	0.8364
5[a]	0.4662	0.4386	0.7563	0.4687	0.4320	0.8219
6[a]	0.4762	0.4840	0.4647	0.4908	0.4667	0.5088
7[b]	0.4840	0.4714	0.6880	0.4847	0.4620	0.7369

VALUES FOR SAMPLE 1 AFTER STORAGE

	Amber Glass	Green Glass	Lightproof Container
x	0.4729	0.4700	0.4863
y	0.5045	0.5077	0.4935
Y/Y_0	0.4018	0.3989	0.3574

[a] Commercial samples.
[b] Sample prepared with a double aqueous maceration.

method. The first sample was divided into four portions and the portions were placed in flint, amber, and green glass, and in lightproof containers. The other three samples were placed in flint glass containers. In addition, two commercial samples of peppermint spirit, U. S. P., were studied. Tristimulus values obtained before and after storage for these samples are given in Table I.

To determine whether a more complete removal of the red and brown colored extraneous substances from the peppermint leaves would give more stability to the color of the spirit, another sample was prepared following the official recipe except that the leaves were macerated for one hour in each of two separate portions of distilled water and then dried overnight before macerating them in the alcohol. Values for this sample are also given in Table I. In this and in all of the other tables the x and y values indicate the color hue in the tristimulus method, and the Y/Y_0 values represent the luminosity of the color.

TABLE II.—TRISTIMULUS VALUES OF SAMPLES PREPARED WITH GUINEA GREEN B[a]

	Initial Values	Final Values after Storage: Flint Glass	Amber Glass	Green Glass	Lightproof Container
Dye Concentration of 70 mg. per Liter					
x	0.4112	0.4471	0.4453	0.4476	0.4476
y	0.4190	0.4082	0.4087	0.4084	0.4074
Y/Y_0	0.8380	0.9883	0.9897	0.9781	0.9826
Dye Concentration of 630 mg. per Liter					
x	0.2277	0.4388	0.4286	0.4409	0.4298
y	0.4688	0.4121	0.4152	0.4119	0.4152
Y/Y_0	0.3666	0.9257	0.8702	0.9339	0.8797

[a] FD & C Green No. 1.

TABLE III.—TRISTIMULUS VALUES OF SAMPLES PREPARED WITH LIGHT GREEN SF YELLOWISH[a]

	Initial Values	Final Values after Storage: Flint Glass	Amber Glass	Green Glass	Lightproof Container
Dye Concentration of 200 mg. per Liter					
x	0.3827	0.4460	0.3318	0.4432	0.4402
y	0.4312	0.4095	0.4105	0.4103	0.4112
Y/Y_0	0.7308	0.9824	0.9503	0.9666	0.9405

[a] FD & C Green No. 2.

TABLE IV.—TRISTIMULUS VALUES OF SAMPLES PREPARED WITH FAST GREEN FCF[a]

	Initial Values	Final Values after Storage: Flint Glass	Amber Glass	Green Glass	Lightproof Container
Dye Concentration of 80 mg. per Liter					
x	0.3839	0.3903	0.3818	0.3835	0.3847
y	0.4236	0.4228	0.4243	0.4238	0.4239
Y/Y_0	0.7215	0.7369	0.7118	0.7210	0.7305
Dye Concentration of 720 mg. per Liter					
x	0.2154	0.2195	0.2154	0.2182	0.2161
y	0.4487	0.4499	0.4490	0.4499	0.4513
Y/Y_0	0.2397	0.2470	0.2396	0.2467	0.2442

[a] FD & C Green No. 3.

INTERPRETATION OF EXPERIMENTAL DATA

While it is recognized that a given variation in tristimulus values will not always mean the same variations in color, since some portions of the spectrum are more compressed than others, in this study the values are all in the green portion of the spectrum, so that twice as much variation in the tristimulus values means approximately twice as much variation in color. The differences in hue values represent the distances between two points plotted on a rectilinear grid, so the ordinary formulas of analytical geometry can be applied to obtain the distances. To obtain the variation in luminosity, the Y/Y_0 values are subtracted.

In the official samples, the variation in initial hue values had a range of 0.002 to 0.077 and the luminosity variation ranged from 0.004 to 0.104. The changes that occurred after storage in all of the samples prepared with mint leaves are given in Table V. It must be remembered that the commercial samples were not freshly prepared when the initial readings were taken, so that the variation in them is not really comparable to the others.

Before considering the tristimulus values obtained for the samples in which the coal tar dyes were used, a reference point was needed so that a comparison of the color obtained with the dye could be made with the typical color of the official spirit.

TABLE V.—VARIATION IN VALUES OF SAMPLES PREPARED WITH MINT LEAVES

Sample	Hue Variation	Luminosity Variation
Values after Storage in Flint Glass		
1	0.053	0.521
2	0.048	0.131
3	0.073	0.230
4	0.095	0.425
5	0.007	0.066
6	0.023	0.044
7	0.009	0.049

Variation in Values of Sample 1 after Storage

	Amber Glass	Green Glass	Light-proof Container
Hue variation	0.050	0.054	0.033
Luminosity variation	0.076	0.074	0.032

The figures on stability indicate the most stable value with the letters *F*, *A*, and *G* designating whether storage was in flint, amber, or green glass containers.

CONCLUSIONS

In this investigation it was found that samples of peppermint spirit prepared by the official process showed variability both in color hue and in depth of color. Furthermore, these samples showed variations in the stability of both the hue and intensity of color when stored in different types of glass.

The samples prepared with Guinea Green B are a poor match for the color of the official spirit, and the color has poor stability independent of storage conditions. The sample prepared with Light Green SF Yellowish is also a poor match for the official spirit in color and offers no advantage in improved

TABLE VI.—HUE VARIATIONS IN SAMPLES PREPARED WITH COAL TAR DYES

Dye	Difference from Value of "Typical" Spirit	Variation after Storage: Flint Glass	Amber Glass	Green Glass	Light-proof Container
Guinea Green B, 70 mg. per liter	0.096	0.038	0.036	0.038	0.038
Guinea Green B, 630 mg. per liter	0.236	0.219	0.213	0.221	0.209
Light Green SF Yellowish, 200 mg. per liter	0.105	0.067	0.063	0.064	0.061
Fast Green FCF, 80 mg. per liter	0.109	0.006	0.002	0.000	0.001
Fast Green, FCF, 720 mg. per liter	0.254	0.004	0.000	0.003	0.003

TABLE VII.—LUMINOSITY VARIATIONS IN SAMPLES PREPARED WITH COAL TAR DYES

Dye	Difference from Value of "Typical" Spirit	Variation after Storage: Flint Glass	Amber Glass	Green Glass	Light-proof Container
Guinea Green B, 70 mg. per liter	0.410	0.150	0.152	0.140	0.145
Guinea Green B, 630 mg. per liter	0.061	0.559	0.504	0.567	0.513
Light Green SF, Yellowish, 200 mg. per liter	0.303	0.252	0.220	0.236	0.210
Fast Green FCF, 80 mg. per liter	0.294	0.015	0.010	0.001	0.009
Fast Green FCF, 720 mg. per liter	0.186	0.007	0.000	0.007	0.005

This would give a value that indicated how closely the colors matched. The method that was used to obtain this reference point was to determine the median tristimulus values for the samples prepared by the official method and for the commercial samples. The median values were: $x = 0.4612$, $y = 0.5008$, and $Y/Y_0 = 0.4277$. These values are not to be interpreted as meaning that they represent the color that peppermint spirit should be; rather, they merely indicate a point from which measurements were made to see which of the coal tar dyes gave a color that most closely resembled that of the official spirit. These differences from the typical color of the mint spirits as well as the variation in color after storage are given in Tables VI and VII.

In Table VIII the data for all of the samples were combined so that a convenient comparison could be made from sample to sample. The figures on hue and luminosity variation in this table were rounded off to the second decimal place, and were then multiplied by 100 so that relative comparisons could be made by the use of small whole numbers.

TABLE VIII.—SUMMARIZED RESULTS

Sample	Relative Difference between Median Values of Official Samples: Hue	Luminosity	Relative Difference between Initial Values and Values after Storage: Hue	Luminosity
Official	..	..	5*A*	7*G*
Guinea Green B, 70 mg. per liter	10	41	4*A*	14*G*
Guinea Green B, 630 mg. per liter	24	6	21*A*	50*A*
Light Green SF Yellowish, 200 mg. per liter	11	30	6*A*	22*A*
Fast Green FCF, 80 mg. per liter	11	29	0*G*	0*G*
Fast Green, FCF, 720 mg. per liter	25	19	0*A*	0*A*
Double maceration	4	26	1[a]	5[a]

[a] Storage in flint glass only.

stability. The samples prepared with Fast Green FCF on the other hand, although still a poor match for the color of the official spirit, show a much greater stability under all conditions of storage. With this dye there is the possibility that mixtures of a stable yellow coal tar dye with Fast Green FCF could be used to approximate the color hue of the official spirits more closely. The use of this dye offers the greatest promise in the development of a simpler way to color the mint spirits.

Samples prepared by the use of the official method, except that two preliminary aqueous macerations of the leaves were carried out, showed a slight increase in stability of the color over that of the samples prepared by the official process.

REFERENCES

(1) Utech, P. H., *Am. J. Pharm.*, **78**, 273(1906).
(2) Williams, E., *Western Druggist*, **37**, 6(1915).
(3) Griffith, L., and Tice, L. F., *Am. J. Pharm.*, **112**, 265 (1940).
(4) Ohmart, L. M., THIS JOURNAL, *Pract. Pharm. Ed.*, **10**, 222(1949).

The Genetic Basis of the Ketones, Carvone, and Menthone in *Mentha Crispa* L.*

MERRITT J. MURRAY and ROBERT H. REITSEMA†

The molecular structure of carvone, the principal ketone of spearmint, differs from that of menthone, the principal ketone of peppermint, in two distinct respects. This difference is due to an allelomorphic change in a single gene with carvone (*C*) dominant over menthone (*c*). The suggested mode of gene action is that the dominant and recessive allelomorphs determine alternative methods of cyclization of a common antecedent ketone.

A PLANT BREEDING PROGRAM to improve the cultivated varieties of mint requires a basic knowledge of the inheritance of disease resistance (1) and at least the major oil compounds. At the start of this investigation, it was not known whether a spearmint crossed to a peppermint gave hybrids containing carvone, menthone, pulegone, or some combination of these ketones. Nelson (2) had previously shown that peppermint, *Mentha piperita* L., could be hybridized with a wild spearmint, *M. crispa* L. He described the first generation species hybrids as having a spearmint, a peppermint, a rubra (menthone), or a pulegone "stink" odor. While the stink odor is an apt descriptive designation for certain hybrids, the chemical compounds responsible for these odors were unknown.

From the viewpoint of heredity, the variability of these species hybrids could be due to the peppermint parent, the spearmint parent, or to both parents. Information on *Mentha crispa* L., is reported here since it is fertile and relatively easy to hybridize.

Chemical research to be efficient required the distillation of single plants, and a method was devised by Reitsema and Baarman (3). A microchemical assay method was used to determine the relative amounts of the ketones and alcohols from the oil of single plants, while a chromatographic method previously described (4, 5) was used in the identification of the specific ketones under consideration.

EXPERIMENTAL

Major Oil Components.—From Table I, it can be seen that carvone is the major ingredient of spearmint oil. Menthone is likewise the major ketone in peppermint oil. While the oil from mature peppermint plants usually has only 25% menthone, the oil from immature plants has a much higher content. It has long been postulated that menthone

TABLE I.—CHEMICAL COMPOSITION

Major Chemical Components, %	Oil of Spearmint	Oil of Peppermint
Ketone	65 (carvone)	25 (menthone)
Alcohol	5–10	50 (menthol)
Ester	5	5
Terpene hydrocarbons & misc.	20–25	20

* Received February 25, 1954. Published with the approval of the Director of Research, Winship A. Todd, as paper No. 5 of the Plant Breeding Laboratory of the A. M. Todd Company, Kalamazoo, Mich.
† Chief geneticist and chemist, A. M. Todd Company.

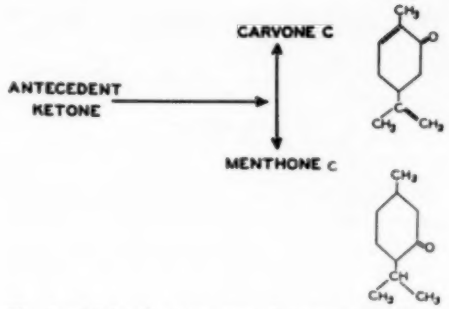


Fig. 1.—Molecular structure of carvone and menthone

is converted into menthol, and indeed the total of the two components is a very constant value in any sample of peppermint oil. The point, which we seek to bring out, is that a ketone is a basic compound in two ways: (*a*) it is one of the major ingredients in these oils, and (*b*) the particular ketone controls the formation of a particular related alcohol and ester. These components account for 75–80% of the oil of peppermint and spearmint.

Chemical Assays.—Carvone and menthone have not been found in the same plant, and there is no reason at this time to believe that they do co-exist. Dihydrocarvone is always associated with carvone, and pulegone with menthone in this material, but it is not our intention here to generalize this statement to include all members of the genus *Mentha*. The details of the chemical work will be published separately. More than a hundred individual plants were assayed; some of these were selected at random, while others were screened for possible differences.

We believe that the samples subjected to careful chemical assay were adequate to delimit the segregation classes for the major ketones under study. After the class standards were established, it was possible to make an accurate classification of the genetic progenies by odor alone. It must be emphasized that other chemical substances occur in *M. crispa*, and that our study of the ketones was primarily qualitative and gave only a rough estimate of quantity.

Inheritance Data—*Mentha crispa* L. is a wild spearmint with very crisp leaves and a poor quality oil by commercial standards. It does not breed true and gives plants which do not have carvone. Our data indicate that a true-breeding spearmint (*CC*) crossed to a true-breeding menthone plant (*cc*) will give all spearmint hybrids (*Cc*). These F_1 spearmint hybrids do not breed true, and their self-pollinated F_2 progeny consists of three spearmints to every nonspearmint plant. There is no reason to suspect that this is not a good 3:1 ratio, since a deviation as great as this would be expected by chance 34 times in every 100 trials.

The F_1 hybrids were also backcrossed to a menthone plant, and the progeny from this cross gave 2072 carvone:2055 menthone individuals. Where $P = 0.8$ and $D/PE = 0.4$, one may consider this a good 1:1 ratio. Several different menthone plants have been progeny tested, and their self-pollinated progeny are all alike in having menthone.

Molecular Structure.—Carvone and menthone differ in two distinct respects in molecular structure as may be seen from Fig 1.

Discussion and Conclusions.—It is of interest to note that an alleomorphic change in a single gene is responsible for the striking and multiple differences in molecular structure. This fact would at first sight seem contrary to the prevalent opinion that one gene produces one enzyme. Such a conclusion is not necessarily warranted, for the problem is one of gene action as well as of gene origin.

From a genetic point of view, it is conceivable that a dominant mutation occurred in a plant having menthone (*cc*) to produce a plant having carvone (*Cc*). It is equally conceivable that a recessive mutation occurred in a plant having carvone (*CC*) to give a menthone-producing plant in subsequent generations. The fact that our allelomorphic difference may have arisen in this manner is not a valid argument that the molecular structure of carvone was directly modified into that of menthone, or that the reverse was true. It appears rather that the allelomorphic differences in a single gene determine alternative methods of cyclization of a common antecedent ketone. We assume that the gene influences a process and only indirectly determines the end product. If this is true, the antecedent ketone should occur as an intermediate product in all these plants regardless of whether they produce carvone or menthone. One should be able to isolate and identify the intermediate and *ontogenetic* compound. This would not be possible, if the antecedent ketone is so highly unstable that it is changed completely and instantaneously into either carvone or menthone.

The concept, that menthone and carvone are alternative modifications of an antecedent ketone, has a sensible phylogenetic basis as we shall show. *Mentha crispa* is a fertile allotetraploid ($2n = 48$) with bivalent pairing, and must have been derived in some manner from diploid species. The suggested double diploid origin of *M. crispa* would indicate that its ketones could have simpler analogs among such diploid species as *M. sylvestris* L. and *M. rotundifolia* L. This hypothesis is under investigation, and we do know that certain strains of *M. crispa* segregate individuals with a musty odor similar to that found in the diploid species *M. sylvestris*.

TABLE II.—INHERITANCE DATA

Ketone	F_2	*B. C.*
Carvone	905	2072
Not carvone	320	2055
Ratio	3:1	1:1
$P.$ =	0.3	0.8
D/PE =	1.4	0.4

REFERENCES

(1) Murray, M. J., *Agron. J.*, in press.
(2) Nelson, R., *Mich. State Coll. Tech. Bull.*, **1950**, 221.
(3) Reitsema, R. H., and Baarman, V. J., THIS JOURNAL, **42**, 734(1953).
(4) Reitsema, R. H., *Anal. Chem.*, **26**, 960(1954).
(5) Reitsema, R. H., THIS JOURNAL, **43**, 414(1954).

Chemical Examination of *Podophyllum emodi* Wall. var. *hexandrum* (Royle)*

By S. C. CHAKRAVARTI and D. P. CHAKRABORTY†

Resin of *Podophyllum emodi* Wall. was isolated. It contains an essential oil responsible for its odor. The resin contains podophyllotoxin (32 per cent), picropodophyllin (0.33 per cent), and six per cent of the yellow coloring matter quercetin. The resin also contains traces of a phenolic compound other than quercetin.

THE RESIN obtained from the American and several Indian species of podophyllum has been subjected to chemical examination and several new compounds have been isolated and their constitutions studied. Some of these compounds and the resin from *P. emodi* and *P. sikkimensis* possess tumor-necrotizing properties. The compounds isolated so far from the three species of Podophyllum are listed in Table I.

TABLE I.—THE CONSTITUENTS OF PODOPHYLLUM RESIN

	P. emodi Wall.	*P. sikkimensis*	*P. peltatum*
1	Podophyllotoxin (1, 2)	Sikkimotoxin (4, 5)	Podophyllotoxin
2	Quercetin (1)	Quercetin (4)	Quercetin
3	4′-Demethylpodophyllotoxin (3)	Iso-rhamnetin (4)	β-Peltatin (6)
4	1-α-(β-D-glucopyranosyl)-picropodophyllin (3)	3-Galactosidyl-quercetin (4)	4′-Demethyl-β-Peltatin (α-Peltatin) (7)
5	Picropodophyllin (1, 3)		

Since *P. emodi* Wall. var. *hexandrum* (Royle) apparently has not been chemically examined before, it was of interest to the authors to examine the nature of the major and the minor constituents present in the resin isolated from the roots and rhizomes.

The roots and rhizomes procured in the month of September from the Kashmir region yielded 7.3 per cent resin. The resin contained 3.68 per cent of a pungent, viscous, brown, essential oil which was responsible for the odor of podophyllin. The major component of the resin appears to be podophyllotoxin (32 per cent), which could be isolated only by fractionation of the resin, involving solvent extraction and, chromatographic adsorption over activated alumina. Six per cent of the resin consisted of the yellow coloring matter quercetin. A small quantity of picropodophyllin (0.33 per cent of the resin) was also isolated from the resin. Picropodophyllin has also been isolated from *P. emodi* (3) both in the free state and as its glucoside and as such it may not be an artifact. Though the presence of a phenolic compound other than quercetin was detected, it could not be isolated in a pure state.

EXPERIMENTAL

The roots and rhizomes of *P. emodi* Wall., var. *hexandrum* (Royle) were procured during the months of September from the Kashmir region through the courtesy of the Divisional Forest Utilization Officer, Baramulla, Kashmir.

The powdered roots and rhizomes (1 Kg.) were extracted in a Soxhlet with dry petroleum-ether (b. p. 60–80°) for forty-eight hours, when the extract became colorless. The yellow-colored extract (6 L.) was separated from the rhizomes and allowed to stand in a refrigerator for twenty-four hours and subsequently cooled to −2° in the freezing mixture for fifteen to twenty minutes when a small quantity (0.30 Gm.) of a pale-yellow, crystalline solid separated, which was filtered off.

Treatment of the Petroleum-Ether Extract.—*Isolation of the Oil.*—On removal of the solvent, a viscous brown pungent oil was left which is responsible for the odor of podophyllum resin. The yield was 0.75% of the dried rhizomes.

The yield of the oil was, however 3.68% of the resin podophyllin. The oil has a refractive index of 1.484 at 35°, the major portion of which distils at 250–280° to a clear liquid with a pungent odor that is irritating to the respiratory system and causes severe headache.

Identification of the White Crystalline Solid: Podophyllotoxin.—The crystalline solid was identified as podophyllotoxin by preparation of monoacetylpodophyllotoxin, m. p. 207–208° and picropodophyllin, m. p. 226–227°.

Fractionation of Podophyllin: Solvent Extraction and Chromatography.—The resin podophyllin (15 Gm.) obtained from the rhizomes according to B. P. 1948, was dissolved in absolute ethanol (20 cc.) by slight warming. Dry benzene (160 cc.) was added to the alcoholic solution and the mixture allowed to stand at room temperature (29–30°) for seven hours when a considerable quantity of heavy tar collected at the bottom.[1] The clear deep brown

* Received December 30, 1953, from the Department of Chemistry, Presidency College, Calcutta, India.
† The authors are indebted to Professor R. Chatterjee for posing the problem and guidance, and to Professor P. C. Rakshit for laboratory facilities.

[1] In two such operations, the separated tar weighed 2.5 Gm. and 2.6 Gm., respectively, accounting for 16.67% and 17.32% of the resin.

solution was separated from the tar and the solvent removed on the water bath. The sticky, brown residue was dissolved in absolute ethanol (40 cc.), and anhydrous benzene (40 cc.) was added to the solution. This solution was utilized for chromatography.

The apparatus for chromatographic adsorption consisted of a tube (2 cm. in internal diameter) fitted at its lower end with a small separating funnel carrying a stopcock. A measuring cylinder was fitted underneath. Aluminum oxide for chromatography prepared by L. Light & Co., and purchased from Nadia Chemical Works, Ltd., of Calcutta, was used. The column was prepared by suspending the adsorbent in anhydrous petroleum ether and permitting it to settle by gravity. The process was repeated till a uniformly packed column (10 cm. in height and 2 cm. in diam.) was produced. The column was washed three times with a cold mixture of absolute ethanol and benzene (1:1 by volume). No suction was employed. The solution of the resin as prepared before (40 cc.) was fed each time through the column (10 cm. x 2 cm.) and the colorless filtrate collected at the bottom. A deep brown zone immediately formed at the upper end. The colorless filtrate (40 cc.), produced no coloration with ferric chloride solution, and was evaporated to crystallization yielding a small quantity of crystals of podophyllotoxin, m. p. 116–118°. These were identified by acetylation to acetylpodophyllotoxin, m. p. 207–208°.

On development, the brown zone considerably lightened in color and descended down the column until 4.2 cm. of the column from the top was colored uniformly pale brown. The color of the first fraction collected progressed from colorless to pale yellow. When the yellow color began to turn definitely brown, collection for the second fraction began. When the brown color gave way to green, the third fraction was collected. The last two fractions though collected separately were entirely colorless. The results are given in Table II.

TABLE II.—CHROMATOGRAPHIC RESULTS

Fraction	Color of the Filtrate	Volume of the Filtrate, cc.	Color Reaction with Ferric Chloride
A	Pale yellow	17	No characteristic color
B	Yellowish brown	25	Green color
C	Pale green	28	No characteristic color
D	Colorless	25	No characteristic color
E	Colorless	25	No characteristic color

Fraction A.—This fraction was concentrated slightly on the water bath and was then allowed to stand for sixty hours at room temperature, at the end of which beautiful transparent prisms separated. The crystals were filtered, washed with a little benzene, and dried. Weight 0.6 Gm., m. p. 177–179°, $[\alpha]_D^{30°}$—129°. The crystals were identified to be anhydrous podophyllotoxin (m. p. of the anhydrous form 179° (8), 183.4° (9), $[\alpha]_D^{30°}$—132°). On acetylation with acetic anhydride alone an acetyl derivative was produced, m. p. 208–209°, which showed no depression on determining the mixed melting point with an authentic sample of acetylpodophyllotoxin, m. p. 208–209°. On refluxing the ethanol solution with fused sodium acetate for ten hours, picropodophyllin, m. p. 227–228° was produced which showed no depression in melting point (226–227°) of an authentic sample of picropodophyllin.

Fraction B.—The solution was slightly concentrated on the water bath and cooled to ordinary temperature, and a small quantity of pale-yellow, crude crystals separated almost immediately. These were filtered off and washed with a little ethanol. The crystals melted indefinitely between 218–225° and gave no color reaction with alcoholic ferric chloride. On crystallizing further from absolute ethanol in which it was sparingly soluble (charcoal), silky needles, m. p. 226–227° were produced. There was no depression in melting point with an authentic sample of picropodophyllin, m. p. 226–227°, yield 25 mg. The bulk of the mother liquor was passed through charcoal, filtered, and concentrated again on the water bath. It was treated with dry benzene and set aside for crystallization and white needles, m. p. 116–118°, were deposited. The mother liquor on concentration and further crystallization gave a second yield of crystals m. p. 116–118°, total yield 1.4 Gm. The crystals were identified to be podophyllotoxin by preparing the acetyl derivative as described before.

Fraction C.—On similar crystallization, podophyllotoxin, m. p. 116–118°, crystallized, and was identified as described above. Weight, 0.4 Gm. Total weight of podophyllotoxin, 2.4 Gm. Yield 32% of the resin.

Fractions D and *E* gave a negligible quantity of solids.

The resin podophyllin isolated from the rhizomes was subjected to analysis according to Dunstan and Henry (1) and it was found that the yield of podophyllotoxin isolated by this method (26%) was the same whether the defatting was done by extraction with petroleum ether either before or after total extraction of the resin. Quercetin was isolated according to the same method to an extent of 6% of the resin.

REFERENCES

(1) Dunstan, W. R., and Henry, T. A., *J. Chem. Soc.*, **73**, 209(1898).
(2) Borsche, W., and Niemann, J., *Ann.*, **499**, 59(1932).
(3) Nadkarni, M. V., Maury, P. B., and Hartwell, J. L., *J. Am. Chem. Soc.*, **74**, 280(1952); and Nadkarni, M. V., Maury, P. V., Hartwell, J. L., and Leiter, J., *ibid.*, **75**, 1308 (1953).
(4) Chatterjee, R., and Dutta, D. K., *Indian J. Physiol. and Allied Sci.*, **4**, 61(1950).
(5) Chatterjee, R., and Chakravarti, S. C., THIS JOURNAL **41**, 415(1952).
(6) Hartwell, J. L., and Detty, W. E., *J. Am. Chem. Soc.*, **72**, 246(1950).
(7) Hartwell, J. L., Schrecker, A. W., and Greenberg, G. Y., *ibid.*, **74**, 6285(1952).
(8) Toms, H., and Pupko, E., *Arb. pharmaceut. Inst. Univ. Berlin*, **13**, 110(1927) (through *Chem. Abstr.*).
(9) Hartwell, J. L., and Schrecker, A. W., *J. Am. Chem. Soc.*, **73**, 2909(1951).

The Comparative Anticholinergic Activity of a Series of Derivatives of 3-Hydroxy Piperidine*

By JOHN P. LONG and HUGH H. KEASLING

Fourteen 3-hydroxy piperidine analogs have been compared to atropine and similar drugs for anticholinergic activity in six tests involving dog, rat, and rabbit. Two of these analogs, JB-323 (N-ethyl-3-hydroxy piperidine benzilate methobromide) and JB-340 (N-methyl-3-hydroxy piperidine benzilate methobromide), are approximately as active as atropine. The 3-hydroxy piperidines, in general, are relatively more active on the intestinal preparations than in inhibiting acetylcholine depressor responses, pilocarpine induced salivation, or β-methacholine induced lacrimation.

Recently a series of analogs of various pharmacologically active agents has been synthesized utilizing 3-hydroxy piperidine as the amino alcohol (1). The present report presents in detail a comparison of 3-hydroxy piperidine derivatives which structurally resemble various active anticholinergic agents.

MATERIALS

The 3-hydroxy piperidines utilized in this study are listed in Fig. 1 with the code designations which will be used in this report. These materials were kindly supplied by Dr. H. L. Daiell of Lakeside Laboratories, Inc. Atropine sulfate ($AtSO_4$), scopolamine (scop. HBr), and homatropine hydrobromide (homat. HBr) were utilized as standards in the various pharmacologic tests. These agents were dissolved in 0.9% saline just before each experiment, with the exception of JB-359, which was dissolved initially in solutions of 10 mg./cc. in 15% ethanol–0.9% saline and diluted with 0.9% saline before use. Acetylcholine chloride (ACh) and β-methacholine chloride were prepared in stock solutions containing 100 mg. compound and 100 mg. KH_2PO_4 in 100 cc. of 0.8% saline (2) and diluted with 0.9% saline for use. Pilocarpine nitrate was utilized as a 5% solution in distilled water.

METHODS

Dog Blood Pressure.—Seventeen dogs were anesthetized with sodium pentobarbital (36 mg./Kg. intravenously) and prepared for recording of either carotid or femoral arterial blood pressure utilizing a mercury manometer writing on a smoked kymograph. All drugs were injected in a constant volume of 1 cc. via a polyethylene catheter inserted into a branch of the femoral vein and washed in with 1 cc. 0.9% saline.

ACh, in geometrically increasing doses, was injected to obtain at least three control depressor responses in the range of 5–20% fall in blood pressure. Following administration of the anticholinergic agent, ACh depressor responses were again obtained via the same geometric progression of dose. The doses following a drug were compared with control and by inspection (in some cases the data were plotted) the shift in ACh sensitivity was determined and recorded as fold increase (e. g., following a drug 1 μg. ACh gave a response equivalent to 0.5 μg. ACh control—twofold increase) (see Fig. 2).

Usually four determinations were carried out in each dog, spaced so that the animal received no injections for a minimum of one hour or until the ACh response level returned to control sensitivity, whichever was longer. The more active compounds were studied in at least three different dogs.

Dog Intestine.—A series of fourteen dogs were anesthetized with sodium pentobarbital (36 mg./Kg., i. v.) and the abdominal wall incised at the midline. The small intestine was carefully lifted from the abdominal cavity in segments of 6–12 inches in length and placed on a saline-soaked towel draped over the abdomen. A polyethylene catheter was tied into a small branch of the mesenteric artery at a Y, so that the tip of the catheter was just short of the branching. Injections into this catheter were swept by the blood stream down the other branch of the Y. Acetylcholine in doses ranging from 0.1 to 1 μg. injected in this manner and washed in with 0.9% saline produced contraction of a segment of intestine 2–4 inches in length. Following insertion of the arterial catheter, a test injection of ACh was made to determine the area affected, then a balloon connected to a water-air tambour was inserted into the lumen of the intestine, and the effect of geometrically increasing doses of ACh was determined. A dose of experimental drug was then given and the ACh sensitivity redetermined. The activity of each compound was expressed as described under *Dog Blood Pressure*. A typical record is shown in Fig. 2. Following a determination, the artery was ligated and the balloon removed and another segment of intestine was prepared. The sensitivity of the various segments of the intestine was not influenced by the dosages of antagonist utilized in this study. Usually five to six satisfactory segments could be obtained from a single dog. A minimum of three segments was utilized for each active agent in different dogs.

Isolated Rabbit Ileum.—Segments of rabbit ileum were suspended in oxygenated Tyrode's solution and the concentration of drug required to give approximately 50% inhibition of maximal acetylcholine-induced spasms determined. All drugs used were dissolved in Tyrode's solution. The compound JB-359 could not be utilized in this study because it was insoluble in Tyrode's solution.

Rat Lacrimation.—The ability of these agents to inhibit the lacrimation induced in rats by the intra-

* Received June 11, 1954, from the Department of Pharmacology, College of Medicine, State University of Iowa, Iowa City, Iowa.

This study was aided by a research grant from Lakeside Laboratories, Inc., Milwaukee, Wis.

CODE	R_1	R_2	X^-	R_3
JB 305	CH_2CH_3	H	Cl	
JB 317	CH_3	H	Cl	
JB 364		H	Cl	
JB 315B	CH_2CH_3	H	Cl	
JB 371	CH_3	H	Cl	
JB 372	CH_3	CH_3	Br	
JB 338	CH_3	CH_3	I	

CODE	R_1	R_2	X^-	R_3
JB 318	CH_2CH_3	H	Cl	
JB 323	CH_2CH_3	CH_3	Br	
JB 336	CH_3	H	Cl	
JB 340	CH_3	CH_3	Br	
JB 344	CH_3	H	Cl	
JB 359	CH_3	CH_3	Br	
JB 355	CH_2CH_3	H	Cl	

Fig. 1.—Structures of 3-hydroxy piperidine analogs with corresponding code numbers.

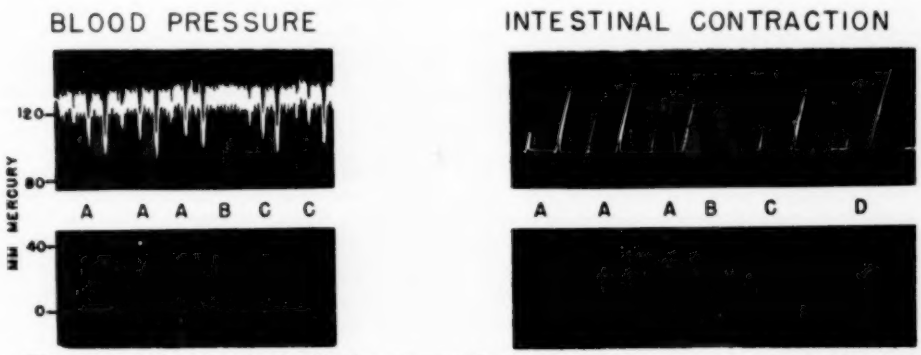


Fig. 2.—Typical tracings for dog blood pressure and intestine. Blood pressure—*A:* 0.5, 1.0, 2.0 μg./Kg. of ACh; *B:* 0.5 μg./Kg. $AtSO_4$; *C:* 1, 2, 4 μg./Kg. of ACh. In this case $AtSO_4$ doubled the dose of ACh, therefore, two-fold increase. Intestinal Contraction—*A:* 0.2, 0.4 μg. ACh; *B:* 0.5μg. JB-340; *C;* 0.4, 0.8, 1.6 μg. ACh; *D:* 0.4, 0.8 μg ACh 5 minutes after *C.*

peritoneal injection of 0.5 mg./Kg. of β-methacholine chloride was determined. Following essentially the method described by Winbury, *et al.* (3), the drugs were dissolved in saline and injected intraperitoneally in volumes of less than 0.4 cc. per rat. For calculating the 50% inhibitory dose, approximately fifteen rats were used to determine each point on the dose response curve.

Rabbit Salivation.—The ability of these agents to inhibit the salivation induced by the intramuscular injection of 5 mg./Kg. of pilocarpine nitrate was determined by modification of the procedure of Brown and Werner (4) in rabbits sedated with 25 mg./Kg. of sodium pentobarbital given intravenously. The experimental drugs were injected intramuscularly three minutes prior to the injection of pilocarpine and the volume of saliva produced in the first ten minutes following pilocarpine was measured. Eight control rabbits produced 23–32 cc. of saliva/10 min.—average 26.2 cc.

Inhibition of Passage of Charcoal Meal.—Essentially the method described by Macht and Barba-Gose (5) was followed. The drugs were injected intraperitoneally in 0.4 cc. of 0.9% saline and ten minutes later the rats were given 1 cc. of 5% charcoal in 5% acacia via a stomach tube. Twenty minutes later, the animals were decapitated, the small intestine removed, and the per cent traverse of the test meal determined. Controls averaged 64%, range (50–71%) in nine animals.

Local Anesthesia, Mydriasis, and Irritation.—The effect of 1% solutions of these agents in 0.9% saline

upon the corneal reflex, pupil size, and local irritation of the rabbit eye was determined in the usual manner.

RESULTS

Dog Blood Pressure.—Table I illustrates the comparative potencies of the various compounds utilizing the various test preparations. $AtSO_4$, JB-323, and JB-340 were essentially equal in activity in this preparation while JB-336 was about one-third as active and JB-318 was approximately 1/25 as active. The remaining derivatives were all much less active. In general, the relatively inactive derivatives gave quite variable shifts in ACh sensitivity and further appeared to be of short duration. With respect to the more active agents, sensitivity usually returned to control with the disappearance of the effect of the agent on ACh depressor responses. Although we did not specifically study duration of action, the impression was gained that JB-340 was relatively short in action compared to JB-323 or $AtSO_4$.

Dog Intestine.—In this preparation the effects of the antagonists are quite variable when more than a 6–8 fold increase in ACh sensitivity is involved; accordingly, our comparisons are based on minimal shifts in ACh sensitivity. In addition to $AtSO_4$, scop. HBr was utilized as a basis of comparison. It is to be noted that JB-340 is approximately equal in activity to scop. HBr and that both are more active than $AtSO_4$. JB-323 and $AtSO_4$ are about equal in activity and approximately one-half as potent as scop. HBr and JB-340. On this preparation, JB-318 and JB-336 are approximately one-quarter as active as JB-340 while JB-359 and JB-344 are also quite active. Significant activity was also possessed by JB-305 and JB-338. The remaining derivatives are relatively inactive.

Isolated Rabbit Ileum.—Comparisons of the dose required for each of these derivatives to produce approximately 50% decrease in maximal ACh-induced spasm reveals that $AtSO_4$, scop. HBr, JB-340, and JB-323 are about equal in activity, requiring 0.5 μg./30 cc. bath volume. JB-336 was approximately one-half as active while JB-318 and JB-344 were one-tenth as active. The remaining derivatives were less than 1/100 as active as $AtSO_4$, although homat. HBr, JB-305, JB-315B, JB-317, JB-338, and JB-364 all varied in this approximate range (1/100–1/200 $AtSO_4$). JB-372 was twice as active as JB-371 although even JB-372 was only 1/1,000 $AtSO_4$.

Rat Lacrimation.—The widest spread in activity among the various active derivatives was obtained in using this procedure. In this test $AtSO_4$ was five times as active as scop. HBr and twenty times as active as JB-323, the most active of the 3-hydroxy piperidine analogs. JB-340 was approximately one-half active as JB-323 while homat. HBr, JB-336, JB-344, and JB-359 were approximately one-tenth as active as JB-323. JB-315B, JB-318, and JB-338 were effective at the highest dose tested while the remaining analogs were relatively inactive at 20 mg./Kg. The duration of action of the active 3-hydroxy piperidine derivatives seemed to be related to potency and did not differ appreciably from the duration of $AtSO_4$, scop. HBr or homat. HBr.

Rabbit Salivation.—JB-323 and JB-340 were the most active materials in this test, while $AtSO_4$ was one-half as active. JB-359, JB-364, JB-344, and JB-336 were one-eighth as active as JB-323 or JB-340. The activity falls markedly for the remaining derivatives and they are not more than one-sixteenth as active as JB-323 or JB-340.

Charcoal Meal.—In this test, all of the compounds exhibited some activity at the dosage levels utilized, although only four of the compounds exhibited marked activity. The most potent agent in this test was JB-323 which was twice as active as $AtSO_4$. JB-340 was one-quarter as active as JB-323, while JB-336 was one-tenth as active. The remaining compounds were no more than one-twentieth as active as JB-323.

Local Anesthesia, Mydriasis, and Irritation.—One per cent solutions of the 3-hydroxy piperidine derivatives, $AtSO_4$ and homat. HBr were compared in the rabbit's eye. Only $AtSO_4$ and homat. HBr produced mydriasis following topical application. None of these materials induced depression of the corneal reflex with the exception of JB-315B. This was so irritating to the eye that the rabbits

TABLE I.—ANTICHOLINERGIC ACTIVITY ON VARIOUS PREPARATIONS

	Dog Blood Pressure, μg./Kg. for 2–4-fold Increase	Dog Intestine, μg./Kg. for 2–4-fold Increase	Rat Lacrimation, mg./Kg. for 50% Inhibition	Rabbit Salivation, mg./Kg. for 60–80% Inhibition	Rat Charcoal Meal, mg./Kg. for 60–80% Inhibition
$AtSO_4$	1.0	1.0	0.10	0.25	1.0
JB-305	500	25.0	20	2.0	10.0
JB-315B	600	200	15	2.0	10.0
JB-317	1000	100	20	2.0	10.0
JB-318	25	2.5	20	2.0	10.0
JB-323	1.0	1.0	2.0	0.125	0.5
JB-336	3.0	2.0	8.0	1.0	5.0
JB-338	500	25	20	2.0	10.0
JB-340	1.0	0.5	5.0	0.125	2.0
JB-344	300	10.0	10.0	1.0	10.0
JB-355	500	100	20	2.0	10.0
JB-359	100	5.0	15.0	1.0	10.0
JB-364	1000	200	20	1.0	10.0
JB-371	600	100	20	2.0	10.0
JB-372	200	100	20	2.0	10.0
Homat. HBr	...	...	7.5	...	...
Scop. HBr	...	0.6	0.5	...	...

squealed in pain following application of this agent. JB-318, JB-340, and JB-344 produced slight irritation, while the remaining derivatives were inactive in this regard.

DISCUSSION

Quaternarization of the piperidyl nitrogen increases activity—compare JB-372 *vs.* JB-371; JB-323 *vs.* JB-318 or JB-336; JB-340 *vs.* JB-336. The effect of methyl *vs.* ethyl on nitrogen in tertiary derivatives is equivocal in that the methyl derivative JB-336 is more active than JB-318 while the ethyl derivative JB-305 is more active than JB-317. Among the various ester groups, the benzilates are by far the most active—compare JB-318, JB-323, JB-336, JB-340 *vs.* the remaining analogs. Furthermore, the substitution of 2-thiophene for phenyl in the benzilate group decreases activity—compare JB-344 *vs.* JB-336. Similarly, in the diphenylacetate series the substitution of cyclohexyl for phenyl decreases activity—compare JB-315B *vs.* JB-305. This change also produces a derivative which is markedly irritating upon topical application. It is difficult to make further generalizations in this regard since the remaining derivatives were all relatively inactive particularly with regard to their actions in the blood pressure, rabbit salivation, and rat lacrimation tests. Although it appears that the diphenylacetates JB-305, JB-315B, JB-317, and JB-364 are about on par with the dibutoline analog, JB-338. It is of interest that the diphenyl methyl ether, JB-359, appears to have considerable activity in all tests, being particularly effective in the rabbit salivation test.

It is of some interest that JB-305, although relatively ineffective in all of the pharmacologic procedures utilized in this study, with the exception of effects on dog intestine, has been reported as a desirable spasmolytic drug for use in man (6).

If one considers as favorable properties of an antispasmodic agent a high activity in the various intestinal preparations relative to activity on blood pressure, salivation or lacrimation, the 3-hydroxy piperidines generally appear more desirable than atropine. In the most active derivatives, JB-323 and JB-340, this is evidenced only in the rat, while for the remaining analogs it is, in general, apparent in each intraspecies comparison.

SUMMARY

1. Fourteen 3-hydroxy piperidine analogs have been compared to atropine and similar drugs for anticholinergic activity in six tests involving dog, rat, and rabbit.

2. Two of these analogs, JB-323 (N-ethyl-3-hydroxy piperidine benzilate methobromide) and JB-340 (N-methyl-3-hydroxy piperidine benzilate methobromide), are approximately as active as atropine.

3. The 3-hydroxy piperidines, in general, are relatively more active on the intestinal preparations than in inhibiting ACh depressor responses, pilocarpine induced salivation or β-methacholine-induced lacrimation.

REFERENCES

(1) Biel, J. H., Friedman, H. L., Leiser, H. A., and Sprengeler, E. P., *J. Am. Chem. Soc.*, **74**, 1485(1952).
(2) Burgen, A. S. V., *Brit. J. Pharmacol.*, **4**, 185(1949).
(3) Winbury, M. M., Schmalgemeier, D. M., and Hambourger, W. E., *J. Pharmacol. Exptl. Therap.*, **95**, 53(1949).
(4) Brown, B. B., and Werner, H. H., *Ibid.*, **97**, 157(1949).
(5) Macht, D. I., and Barba-Gose, J., THIS JOURNAL, **20**, 558(1931).
(6) Weinberg, B., Ginsberg, R., and Sorter, H., *Am. J. Digest. Dis.*, **20**, 230(1953).

Substituted Xanthines. I. Preparation and Properties of Some Choline Theophyllinates*

By B. F. Duesel, H. Berman, and R. J. Schachter

Choline was condensed with theophylline and 8-substituted theophylline to give a series of well-defined, water-soluble theophylline derivatives. Choline theophyllinate was found to be a safe and effective drug.

THE XANTHINES, particularly theophylline alone or with the addition of ethylene diamine (aminophylline), have long been used as therapeutic agents mainly to cause diuresis and to relieve the brochospasm associated with asthmatic phenomena. Other properties of the xanthines such as vasodilation, either of the coronaries or peripheral blood vessels, have been reported in recent years.

Since theophylline and aminophylline cause gastric irritation with concomitant nausea and vomiting, the clinical use of these compounds is limited. In addition, theophylline is quite insoluble in water. With this in mind a series of water-soluble theophylline compounds was prepared which might be devoid of irritating properties.

* Received March 31, 1954, from the Research Laboratories, Nepera Chemical Company, Inc., Yonkers 2, N. Y.

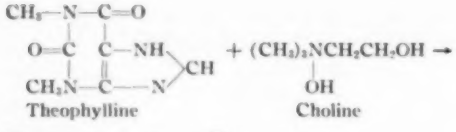
TABLE I.—SOLUBILITY OF COMPOUNDS

Compound	Solubility at Room Temperature, Gm. : ml. Water	pH of 0.8% Aqueous Solution
Theophylline	1:120	4.8
Choline theophyllinate[a]	1:1	9.7
Choline 8-chloro-theophyllinate	1:1	7.9
Choline 8-bromo-theophyllinate	1:1	8.7
Choline 8-nitro-theophyllinate	1:1.5	7.3

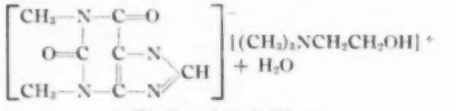
[a] Choledyl ® is the registered trade mark of Nepera Chemical Company, Inc., for its brand of choline theophyllinate.

Table I gives the solubility of the compounds at room temperature and the *p*H of an 0.8 per cent aqueous solution.

In this study it was observed that the resulting derivatives were not merely addition compounds but salts, formed by the splitting out of water between the choline and theophylline moieties as follows:



Theophylline Choline



Choline theophyllinate

The compounds were prepared by several methods. In early runs, choline base in alcohol was prepared by the addition of alcoholic potassium hydroxide solution to an alcoholic solution of choline chloride (1). After removal of the precipitated potassium chloride, the solution of choline base was added to an alcoholic suspension of the desired theophylline compound. The mixture was refluxed for a short time, after which the product crystallized out on cooling. In some instances it was necessary to concentrate the alcohol or add a second solvent such as benzene to precipitate the reaction product. Final purification of the product was effected by a crystallization from alcohol or a mixture of alcohol and benzene. While this procedure was satisfactory for laboratory work, it left much to be desired for large-scale operation.

The method of choice[1] was found to be a procedure wherein the desired theophylline intermediate was warmed with a slight excess of a 45–50% aqueous choline bicarbonate solution until the evolution of carbon dioxide ceased. The water content was reduced azeotropically by the addition of 2-propanol followed by distillation under vacuum.

[1] U. S. patent pending.

PHARMACOLOGY

Choline theophyllinate was subjected to the usual laboratory pharmacological studies, such as toxicity both acute and chronic, the effect on blood pressure and relief of bronchospasm.

Toxicity.—The acute toxicities of choline theophyllinate have been determined in mice, guinea pigs, and rats. For mice and guinea pigs the following routes were used: oral, intramuscular, and intravenous. In rats, oral, intramuscular, and intraperitoneal toxicities were determined. The results of these studies are given in Table II.

TABLE II.—THE ACUTE TOXICITIES OF CHOLINE THEOPHYLLINATE IN MICE, GUINEA PIGS, AND RATS

Species	Route	Dose, mg./Kg.	Number Dead/ Used	LD_{50}, mg./Kg.
Mouse	Oral	500	0/20	
		600	4/20	
		700	7/20	
		800	12/20	770
	i. m.	200	0/20	
		300	2/20	
		350	7/20	
		375	17/20	
		400	20/20	360
	i. v.	75	0/20	
		88	4/20	
		100	7/20	
		125	13/20	
		150	26/30	112
Guinea pigs	Oral	100	0/10	
		175	0/10	
		188	2/10	
		200	6/10	
		250	8/10	
		300	9/10	
		500	9/10	210
	i. m.	100	0/10	
		150	2/10	
		200	5/10	
		225	9/10	
		250	10/10	185
	i. v.	75	0/10	
		100	1/10	
		125	7/10	
		150	8/10	
		200	10/10	118
Rats	Oral	400	0/20	
		500	3/20	
		600	11/20	
		700	15/20	
		800	18/20	600
	i. p.	100	0/10	
		150	1/10	
		175	2/10	
		200	15/20	
		300	16/20	
		400	16/20	185
	i. m.	150	0/10	
		200	2/10	
		250	6/10	
		300	8/10	
		325	10/10	240

TABLE III.—LD_{50} OF THEOPHYLLINE COMPOUNDS

Compound	mg./Kg.
Choline theophyllinate	770
Choline chlorotheophyllinate	*Ca.* 500
Choline nitrotheophyllinate	5000 (not lethal)
Choline bromotheophyllinate	*Ca.* 850
Theophylline	600
Aminophylline	585

For comparative purposes the acute oral toxicities of several theophylline compounds were determined in mice. The LD_{50} of these compounds are given in Table III. The LD_{50} were calculated according to the graphic method of Litchfield and Wilcoxon (2).

Choline theophyllinate was fed to growing rats for ten months and adult dogs for six months in amounts as high as 300 mg./Kg./day. At the end of the experimental period the rats had normal growth and clinically were indistinguishable from normal rats. The dogs gained or maintained their weight and to all appearances were normal. The hematology of both rats and dogs was normal. Necropsy and histology presented normal tissues.

Circulation and Respiration.—Intravenous doses of choline theophyllinate (6–10 mg./Kg.) caused negligible changes in carotid blood pressure of anesthetized dogs; however, the same amount of choline theophyllinate gave prompt relief to neostigmine-induced bronchospasm in anesthetized dogs.

The above data indicate that choline theophyllinate is a safe and effective therapeutic agent.

EXPERIMENTAL[2]

Choline Theophyllinate.—Eighteen grams (0.1 mole) of anhydrous theophylline was added to 38.7 Gm. of an aqueous choline bicarbonate solution assayed at 47% (10% excess). The mixture was stirred and heated between 80–90° until the evolution of carbon dioxide had ceased and complete solution had been effected. In some instances, a precipitate of the desired compound came out shortly after solution had resulted. Fifteen milliliters of water was distilled over, using sufficient vacuum to keep the still temperature between 50–55°. A volume of 100 ml. of 2-propanol was then added and 50 ml. of 2-propanol-water distillate was collected *in vacuo*. A white crystalline solid precipitated out during this operation. After allowing the reaction mass to cool slowly to room temperature, the mixture was chilled to +5°. The white crystalline solid was filtered off, pressed well, and washed twice with 5-ml. portions of 2-propanol. After drying at 70° in vacuum, the product weighed 21.8 Gm. or 77% of theory. Another 15 ml. of 2-propanol was removed from the filtrate and a second crop weighing 4 Gm. was obtained which gave a total yield of 90.5%. The product melted at 186° (corr.).

Anal.—Calcd. for $C_{12}H_{21}N_5O_5$: N, 24.8; C, 51.0; H, 7.47. Found: N, 24.6; C, 51.3; H, 7.53.

Choline 8-Bromotheophyllinate.—8-Bromotheophylline was prepared by the method of Biltz and Strufe (3). To 37 Gm. of a 45% solution of choline bicarbonate (0.1 mole) was added 25.9 Gm. (0.1 mole) of bromotheophylline under vigorous stirring. Carbon dioxide was evolved and the addition was carried out as rapidly as possible without loss. The mixture was then warmed to 40° to complete the reaction which required about one-half hour. A small amount of insoluble material was filtered off and the clear solution diluted with 100 ml. of 2-propanol. Seventy-five milliliters of 2-propanol-water mixture was distilled over, using a vacuum of about 20 mm. Hg. The still temperature did not exceed 50° during this operation. Another 100 ml. of 2-propanol was added and 75 ml. distilled over as before. Fifty milliliters of benzene was then added and distillation in a vacuum continued until the reaction mixture was no longer turbid. On cooling slowly to 45°, white crystals began to separate out. After standing overnight the mixture was cooled to 5° and the crystals filtered off and washed twice with 5-ml. portions of benzene-2-propanol mixture (two parts of benzene to one of alcohol). When the bulk of the solvent had been removed by drying in a vacuum oven at room temperature, the temperature was raised to 40° and drying continued to constant weight until 29.2 Gm. was obtained, or 80.5% of theory. A second crop was obtained by concentrating the filtrate, giving a total yield of 90%. The crystalline product obtained under these condition contains water of crystallization equivalent to about 9% as determined by the Karl Fischer method which corresponds to a di-hydrate, m. p. 67°. When dried at 60° to constant weight the product was an amorphous hygroscopic solid.

Anal.—Calcd. for $C_{12}H_{20}N_5O_5$-Br: N, 19.3. Found: N, 18.9.

8-Nitrotheophylline.—Directions for preparing 8-nitrotheophylline are given in a P. B. report (4). The best yield that could be obtained following these directions was about 20%. When carried out as directed the procedure was hazardous as the reaction could not be properly controlled when more than small amounts were run. The following procedure was found to be readily controllable and gave a 65.5% yield, even when large batches were prepared.

Five hundred grams of anhydrous theophylline was added to 1 L. of glacial acetic acid in a 5-L. three-neck flask fitted with a stirrer, condenser, and thermometer. A thick slurry resulted which upon heating to 87° became fluid. Four hundred ninety grams of conc. nitric acid was added dropwise. The first 15 ml. resulted in a vigorous reaction taking place with the violence tapering off shortly thereafter. The addition of nitric acid was completed in about one hour. During this operation the temperature was controlled between 87° and 110° by the rate of addition of the nitric acid. The reaction mass was cooled in an ice bath to 5° and the canary-yellow solid filtered and washed with 2 L. of water to free it from acetic acid. Four hundred eighty grams of the di-hydrate melting at 282–283° was obtained. This is a 66.5% yield.

Choline 8-Nitrotheophyllinate.—8-Nitrotheophylline, 5.2 Gm., (0.02 mole) was added to 7.74 Gm. choline bicarbonate solution assayed at 47% (10% excess over theory). On warming to 60° a clear yellow solution resulted within a half hour during which time carbon dioxide was evolved. Fifteen milliliters of ethanol was added to the mixture while still hot. Canary-yellow crystals of the

[2] The analyses were carried out by Miss Ruth Becker.

product separated out. When cooled to room temperature the crystals were filtered off and washed with ethanol. The product was recrystallized from 150 ml. of 90% ethanol. The yield of recrystallized material was 5.4 Gm. or 85% of theory. The product melted at 248°.

Anal.—Calcd. for $C_{12}H_{20}N_6O_5$: N, 25.6. Found: N, 25.4.

Choline 8-Chlorotheophyllinate.—215.5 Gm. (1.0 mole) of 8-chlorotheophyllinate was slowly added to 370 Gm. of choline bicarbonate solution (47% assay) under stirring, keeping the temperature at 50°. The addition was completed in one hour and the temperature was raised to 60° for half an hour. All was in solution with a brown color. One liter of 2-propanol was added to the mixture and 800 ml. distilled over to carry over part of the water. The operation was repeated using one more liter of 2-propanol and removing 800 ml. Six hundred milliliters of benzene was then added to the mixture and 200 ml. distilled over. On cooling, clusters of white needles came out. After chilling at 5° for an hour the crystals were filtered off and washed with a mixture of three parts benzene and one part of 2-propanol. The product was dried in a vacuum at 40°. One hundred ninety-eight grams of product was obtained in the first crop and 38.6 Gm. by further evaporation of the filtrate. The pure material melts at 60–62°. Analysis for water by the Karl Fischer method indicated it was a di-hydrate. On drying to constant weight at 60° the melting point rose to 97–99° and analysis indicated it was the anhydrous material.

Anal.—Calcd. for $C_{12}H_{20}ClN_5O_3$: N, 22.05. Found: N, 21.13.

SUMMARY

(1) The synthesis of a new class of theophylline derivatives is described.

(2) The pharmacology of choline theophyllinate indicates that it is a safe therapeutic agent. Clinical investigation has proved this drug to be an effective xanthine with superior patient acceptance. These findings are reported elsewhere.

REFERENCES

(1) Feinstone, W. H., U. S. pat. 2,667,487, 1953. Feinstone, W. H., and Duesel, B. F., U. S. pat. 2,661,352, 1953.
(2) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).
(3) Blitz, H., and Strufe, K., *Ann.*, **402**, 136(1914).
(4) Bibliography of Scientific and Industrial Reports, **9**, 990(1948). P.B. L-83616.

In vitro Antimicrobial Activity of a Dehydroacetic Acid and *p*-Chlor-*m*-xylenol Combination*

By R. L. STEDMAN†, S. L. ENGEL‡, and I. M. BILSE§

The *in vitro* activity obtained with a new combination of antimicrobial agents, dehydroacetic acid and *p*-chlor-*m*-xylenol is described. Formulations containing these agents show significant activity against fungal, bacterial, and protozoan species commonly associated with superficial mycotic infections and trichmoniasis. In many instances, the combination can be shown to be superior *in vitro* to certain representative products currently available for the treatment of these conditions.

ALTHOUGH superficial mycotic infections, including vaginal mycosis with or without trichomoniasis, have been treated by a number of chemical agents of diverse structure, it is the opinion of many that new, more effective agents are required. This report describes the *in vitro* antimicrobial activity of a combination of *p*-chlor-*m*-xylenol and dehydroacetic acid against organisms of importance in these diseases.

Dehydroacetic acid has been shown to be active against both bacteria and fungi (1) under a variety of conditions. The compound requires relatively high concentrations for inhibition of saprophytic fungi but the dermatophytes are more sensitive. Extensive pharmacological studies have revealed a low systemic toxicity and freedom from irritative or sensitizing properties (2–5). *p*-Chlor-*m*-xylenol is not a new antimicrobial agent. It has been employed as an antiseptic and preservative for a number of years in Great Britain. Recently, interest has been shown in this compound as a bacteriostatic agent in hand soap. It is more effective than dehydro-

* Received February 26, 1954 from the McNeil Laboratories, Inc., Philadelphia, Pa.
† Present address: Industrial Test Laboratory, Philadelphia Naval Shipyard, Naval Base, Philadelphia 12, Pa.
‡ Present address: Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, N. Y.
§ Present address: 1227 West Venango Street, Philadelphia, Pa.

TABLE I.—IN VITRO ACTIVITY OF DHA-PCMX COMBINATIONS

	C. albicans		*T. mentagrophytes*		*M. pyogenes* var. *aureus*	
	MIC[a]	CKD[b]	MIC	CKD	MIC	CKD
DHA	1:3,000	>20%	1:10,000	>5%	1:50	>10%
PCMX	1:10,000	1:150	1:30,000	1:300	1:6,000	1:600
DHA plus PCMX	1:5,000 1:40,000	10% 1:200	1:20,000 1:60,000	5% 1:300	1:200 1:12,000	10% 1:600

[a] Minimum inhibitory concentration; *C. albicans* and *T. mentagrophytes* incubated at room temperature for seven days and fourteen days, respectively, and *M. pyogenes* var. *aureus* incubated at 37° for five days.
[b] Critical killing. Dilution at 37° (kills in ten minutes but not in five minutes).

TABLE II.—INHIBITORY ACTIVITY OF DHA-PCMX SOLUTION AND OTHER PREPARATIONS

Test Organism	Zones of Inhibition (mm. from Pad Edge to Zone Edge)			
	DHA-PCMX Solution	Solution *A*	Solution *B*	Solution *C*
E. inguinale[b]	20.0	8.5	7.0	1.0
M. audouini[b]	C. P.[a]	34.0	14.5	9.0
T. mentagrophytes	29.0	20.0	17.0	6.0
T. purpureum[b]	28.0	16.0	9.0	5.5
C. albicans	20.5	13.0	3.5	5.0
M. pyogenes var. *aureus*	7.0	3.0	5.0	2.5

[a] Clears plate.
[b] Strain number unknown.

acetic acid against bacteria and fungi and is lacking in the irritation and sensitization frequently encountered with some halogenated phenols (6, 7).

EXPERIMENTAL METHODS AND RESULTS

A number of test methods were employed in this study; modifications of procedures were required in some instances. To facilitate presentation, the methods and modifications are indicated in the pertinent sections. In all cases, tests were run at least three times and representative data reported.

The Effects of Combining Dehydroacetic Acid and *p*-Chlor-*m*-xylenol.—Initial tests were designed to determine the antimicrobial effects of combination on the individual activities of the two agents. Aqueous solutions of the sodium salt of dehydroacetic acid (DHA) and *p*-chlor-*m*-xylenol (PCMX) were used in all tests, 30% polyethylene glycol 300 in water being employed as the solvent for PCMX and as the diluent for both agents. Bacteriostatic and fungistatic properties were determined against *Micrococcus pyogenes* var. *aureus* 209, *Candida albicans* (stock strain), and *Trichophyton mentagrophytes* (ATCC 9533) using the Kolmer-Boerner serial dilution technique (8). Bactericidal and fungicidal activities were determined by the phenol coefficient method without phenol dilutions (9). The findings are presented in Table I.

In the inhibitory tests, combination of the agents resulted in lower concentrations of each agent required for the desired effect. In the fungicidal and bactericidal tests, a slight cooperative effect can be demonstrated only against *C. albicans;* no antagonism is observed in the other instances.

These data show that DHA and PCMX are not antagonistic and that a cooperative effect can be demonstrated under certain conditions. The value of combining the agents would appear to lie primarily in the enhancement of inhibition rather than killing.

Comparative Antimicrobial Studies on the DHA-PCMX Combination.—*Against Test Organisms Commonly Associated with Superficial Mycoses.*—A solution of 10% DHA and 2% PCMX in an aqueous solvent containing 30% polyethylene glycol 300 and 15% isopropyl alcohol was prepared. The *in vitro* activity of this solution was then compared to the following commercial products which are available for use in the treatment of superficial mycoses: (*a*) A solution containing 12.3% sodium propionate, 2.7% propionic acid, and 10% sodium caprylate (designated *A*). (*b*) A solution containing 2% each of salicylic and benzoic acids, 5% chlorobutanol, and 1:10,000 malachite green (designated *B*). (*c*) A solution containing 2% undecylenic acid with 5.2% zinc stearate (designated *C*).

Fungistatic and bacteriostatic characteristics of these formulations were determined against a number of organisms using the agar diffusion technique of Kligman and Rosensweig (10) with this slight modification: 1% bovine albumin (Armour) was added to the seed layer, and one pad was used per plate instead of the usual three.

Table II lists the representative data obtained. Most of the preparations show significant activity against the test organisms by this method. The DHA-PCMX combination is the most effective preparation of the group.

The fungicidal activities of the formulations were then determined using the Golden and Oster modification (11) of the Burlingame-Reddish test (12) but with 1% bovine albumin in the inoculum medium. DHA-PCMX solution and solutions *A* and *B* kill the mycelial pads of *T. mentagrophytes* in less than one minute, but solution *C* requires ten minutes' exposure.

In general then, these findings indicate that the DHA-PCMX solution possesses outstanding *in vitro* antimicrobial activity when tested comparatively against certain representative products and when following the indicated procedures.

Against Organisms Associated with Vaginal Mycosis and Trichomoniasis.—An ointment consisting

of 20% DHA and 5% PCMX in a base of equal parts polyethylene glycol 300 and Carbowax® 1400 was prepared and tested comparatively against the following products available on the market for use in the treatment of vaginal mycosis and trichomoniasis: (*a*) A suppository containing approximately 2.6% silver picrate (designated *D*). (*b*) A vaginal tablet having approximately 8.2% 5,7-diiodo-8-hydroxyquinoline and 5.4% boric acid (designated *E*). (*c*) A jelly containing 10% sulfathiazole, 3% lactic acid, and 1% acetic acid (designated *F*).

The inhibitory activity of the formulations against *Candida albicans* and *Trichomoniasis vaginalis*[1] were studied using the Kolmer-Boerner method with 1% bovine albumin added to all tubes subsequently seeded with *C. albicans*. The following changes in procedure were necessitated by the growth characteristics of the protozoan: *T. vaginalis* was grown at 37° in the medium of Kupferberg, *et al.* (13), with 10% human plasma adjusted to *p*H 5.9. Forty-eight hour cultures were checked microscopically for motile organisms before being used as inocula. The same medium was employed in the inhibitory tests. All inhibitory tests were incubated at 37° for three days. At the end of this time, all tubes were examined microscopically for growth and motility. The occurrence of three or more motile cells per field was arbitrarily considered an indication of a lack of inhibition.

Table III gives the inhibitory dilutions of the formulations on a weight-volume basis.

TABLE III.—COMPARATIVE INHIBITORY ACTIVITY OF DHA-PCMX OINTMENT AND OTHER PREPARATIONS

Preparations	Minimum Inhibitory Concentrations	
	C. albicans	*T. vaginalis*
DHA-PCMX	1:800	1:600
D	1:100	1:400
E	1:100	1:400
F	1:100	1:100

TABLE IV.—COMPARATIVE FUNGICIDAL AND TRICHOMONADOCIDAL ACTIVITY OF DHA-PCMX OINTMENT AND OTHER PREPARATIONS

Preparations	*C. albicans*[a]	*T. vaginalis*[b]
DHA-PCMX Ointment	30 min.	1:100–1:200
D	5 min.	1:50 –1:500
E	6 hr.	1:10
F	6 hr.	1:10

[a] Killing time for a 1:2 dilution of preparation.
[b] Ranges of killing dilutions.

Against *C. albicans*, the DHA-PCMX ointment is eight times as effective as any of the others by this method; using *T. vaginalis* as the test organism, DHA-PCMX ointment is also more active than the other products, but the superiority is not as marked in all cases.

[1] The authors wish to thank the Ortho Pharmaceutical Corporation, Raritan, N. J., for supplying the culture of *T. vaginalis*.

Fungicidal and trichomonadocidal tests were then conducted. The phenol coefficient method was employed using *C. albicans* and a modification of this method (14) in tests against *T. vaginalis*. The modified procedure is as follows: 1.0 ml. of an undiluted forty-eight-hour culture of *T. vaginalis* was used as inoculum in each seeding tube; all dilutions of preparations tested contained 1% bovine albumin; 0.1 ml. of the seeding tube contents was withdrawn at five- and ten-minute intervals and inoculated into 10 ml. previously described medium; all subcultures were incubated at 37° for five days and then examined microscopically for growth.

Table IV presents the data obtained in these killing tests. As pointed out by Trussel (14), end points using this method are difficult to reproduce and values are best presented as a killing range.

DHA-PCMX ointment and preparation *D* are the more effective preparations against both organisms. The latter is superior to DHA-PCMX ointment against *C. albicans*. Unfortunately, the wide ranges of killing for these two formulations against the protozoan overlap and do not permit a statement concerning which is the more active under these conditions.

SUMMARY

1. Combinations of dehydroacetic acid and *p*-chlor-*m*-xylenol are active antibacterial, antifungal, and antitrichomonadal agents as tested *in vitro*.

2. Solely on the basis of *in vitro* antimicrobial data, formulations containing these agents appear in most instances to be equal or superior to several representative products now available for use in the treatment of superficial and vaginal mycotic infections and vaginal trichomoniasis.

REFERENCES

(1) Wolf, P. A., and Westveer, W. M., *Arch. Biochem.*, **28**, 201(1950).
(2) Spencer, H. C., *et al.*, *J. Pharmacol. Exptl. Therap.*, **99**, 57(1950).
(3) *Ibid.*, **99**, 69(1950).
(4) *Ibid.*, **99**, 84(1950).
(5) *Ibid.*, **99**, 98(1950).
(6) von Oettinger, W. F., National Institutes of Health Bulletin No. 190 (1949).
(7) Colebrook, L., and Mafted, W. R., *J. Obstet. Gynaecol. Brit. Empire* **40**, 966(1933).
(8) Kolmer, J., and Boerner, F., "Approved Laboratory Technic," 2nd ed., Appleton-Century Co., New York, 1938, pp. 519–520.
(9) Ruehle, G. L. A., and Brewer, C. M., Circular 198, U. S. Dept. of Agric., 1931.
(10) Kligman, A. M., and Rosensweig, W., *J. Invest. Dermatol.*, **10**, 51(1948).
(11) Golden, M. J., and Oster, K. A., THIS JOURNAL, **36**, 359(1947).
(12) Burlingame, R. M., and Reddish, G. F., *J. Lab. Clin. Med.*, **24**, 765(1939).
(13) Kupferberg, A. B., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **67**, 304(1948).
(14) Trussel, R. H., "Trichromonas Vaginalis," Charles C Thomas, Springfield, Ill., 1947.

Piperazines*

IV. 1-Heterocyclic-4-substituted carbamyl- and -thiocarbamylpiperazines

By E. A. CONROY, N. Q. QUINONES, K. L. HOWARD,† H. W. STEWART, and J. J. DENTON

The preparation and anticonvulsant activity of twenty-four new 1-heterocyclic-4-substituted carbamyl- and -thiocarbamylpiperazines are presented. Three were prepared by the reaction of 1-diethylcarbamylpiperazine with the appropriate halogen-substituted heterocycle, while the remaining twenty-one were prepared by the reaction of 1-heterocyclic piperazines with the appropriate isocyanate, isothiocyanate, or substituted carbamyl chloride. Five of these compounds received maximum anticonvulsant rating by the general testing methods employed.

IN THE COURSE of the investigation of 1-heterocyclic-4-substituted-piperazines in this laboratory (1–5), it was noted that several 1-heterocyclic-4-monoalkylcarbamyl- and -monoalkylthiocarbamylpiperazines had pronounced anticonvulsant activity when tested against audiogenic seizures in rats. This observation led to the preparation of the 24 compounds listed in Table I for pharmacological evaluation.

All of the compounds listed in Table I, with the exception of compounds VI, XII, and XXIV, were tested in susceptible rats by the audiogenic-Metrazol® method for the purpose of detecting general anticonvulsant activity. In this method, a convulsion is precipitated by subjecting the animal to a sound stimulus of definite duration after the preliminary administration of a subconvulsant dose of Metrazol®. The percentage of rats protected from convulsions by the compound is indicated by the activity rating. The maximum rating, indicated by plus signs, which a compound may receive by this method is ++++.

Compounds VI, XII, and XXIV were tested by means of the corneal electroshock method (6). In this method, a convulsion is produced by subjecting the rat to an electrical stimulus of definite magnitude and duration by means of electrodes placed upon the corneas. The percentage of rats protected from convulsions by the compound is indicated by the activity rating. The maximum rating which a compound may receive by this method is ++++.

Compounds which exhibited interesting activity in the above general testing procedures were tested further by means of the electroshock method of Merritt and Putnam (7) adapted to the rat. In this method, the electro-convulsant threshold is determined by passing a current through the forebrain of the rat by means of electrodes attached to the ears. The activity rating of a compound is based on the percentage increase in the electro-convulsant threshold of the affected rats. The maximum rating which a compound may receive by this method is ++++.

Five of the compounds in Table I were given maximum activity ratings in the general tests, but none of these which were further tested by the method of Merritt and Putnam showed outstanding activity.

EXPERIMENTAL

Procedure (A).—*Preparation of 1-Heterocyclic-4-monoalkylthiocarbamylpiperazines.*—To a stirred solution of 0.30 mole of the appropriate 1-heterocyclic piperazine in 200 ml. of diethyl ether was added a solution of 0.30 mole of the appropriate alkyl isothiocyanate in 200 ml. of diethyl ether over a period of ten minutes with cooling in an ice bath. The precipitate which formed was removed, recrystallized from benzene or ethyl acetate, and dried.

Procedure (B).—*Preparation of 1-Heterocyclic-4-monoalkylcarbamylpiperazines.*—To a stirred solution of 0.15 mole of the appropriate 1-heterocyclic piperazine in 200 ml. of diethyl ether was added a solution of 0.15 mole of the appropriate alkyl isocyanate in 200 ml. of diethyl ether over a period of ten minutes with cooling in an ice bath. The precipitate which formed was removed, recrystallized from VMP naphtha, and dried.

Procedure (C).—*Preparation of 1-Heterocyclic-4-(N-ethyl-N-phenyl)carbamylpiperazines.*—A suspension of 0.30 mole of sodium carbonate in a solution of 0.30 mole of the appropriate 1-heterocyclic

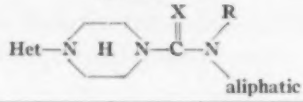
* Received January 5, 1954, from the Research Department, Calco Chemical Division, American Cyanamid Company, Bound Brook, N. J.

† Present address: Wallerstein Laboratories, 180 Madison Avenue, New York, N. Y.

The authors wish to express their appreciation to R. W. Cunningham, B. K. Harned, and Frank Smith of the Lederle Laboratories Division, American Cyanamid Company, for the pharmacological testing.

The microchemical analyses were carried out by O. E. Sundberg, M. E. Nielsen, and I. H. Prokul.

TABLE I.—ANTICONVULSANT ACTIVITY OF SOME 1-HETEROCYCLIC-4-CARBAMYL- AND -THIOCARBAMYL-PIPERAZINES



No.	Het	X	R	Aliphatic	Activity,[a] mg./Kg. Body Weight Audiogenic	Electroshock
I	2-Pyridyl	S	H	Ethyl	++i 150 i. p.	
II	2-Pyridyl	S	H	Allyl	++++ 150 i. p.	
III	2-Pyridyl	O	Ethyl	Ethyl	+i 75 i. p.	
IV	2-Pyridyl	O	H	n-Hexyl	+++i 100 i. p.	± 125 i. p.
V	2-Pyridyl	O	H	Cyclohexyl	++++ 300 i. p.	0 150 i. p.
VI	2-Pyridyl	O	Phenyl	Ethyl	++++[b] 100 i. p.	
VII	2-Pyrimidyl	S	H	Ethyl	++i 225 i. p.	
VIII	2-Pyrimidyl	S	H	Allyl	++++ 180 i. p.	0 200 i. p.
IX	2-Pyrimidyl	O	Ethyl	Ethyl	+i 100 i. p.	
X	2-Pyrimidyl	O	H	n-Hexyl	++i 250 i. p.	
XI	2-Pyrimidyl	O	H	Cyclohexyl	++++ 125 i. p.	± 100 i. p.
XII	2-Pyrimidyl	O	Phenyl	Ethyl	++[b] 10 i. p.	
XIII	5-Chloro-2-pyrimidyl	O	Ethyl	Ethyl	0 15 i. p.	
XIV	2-Pyrazinyl	S	H	Ethyl	0 40 i. p.	
XV	2-Pyrazinyl	S	H	Allyl	++i 200 i. p.	
XVI	2-Pyrazinyl	O	Ethyl	Ethyl	0 50 i. p.	
XVII	2-Pyrazinyl	O	H	n-Hexyl	++ 50 i. p.	
XVIII	2-Pyrazinyl	O	H	Cyclohexyl	0 225 i. p.	
XIX	2-Thiazolyl	S	H	Ethyl	0i 500 i. p.	
XX	2-Thiazolyl	S	H	Allyl	0i 500 oral	
XXI	2-Thiazolyl	O	Ethyl	Ethyl	0 100 i. p.	
XXII	2-Thiazolyl	O	H	n-Hexyl	+++i 180 i. p. ++i 750 oral	0 150 i. p.
XXIII	2-Thiazolyl	O	H	Cyclohexyl	++i 225 i. p. ++i 500 oral	0 250 i. p.
XXIV	2-Thiazolyl	O	Phenyl	Ethyl	++[b] 50 i. p.	

[a] Significance of ratings: The compounds are tested intraperitoneally (i. p.) or orally (oral) at about the highest dose which does not produce symptoms of toxicity. The maximal theoretical protection is ++++. A rating of +++i indicates an activity of between +++ and ++++.

[b] The corneal electroshock method was used instead of the audiogenic method.

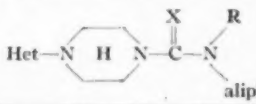
piperazine and 0.30 mole of (*N*-ethyl-*N*-phenyl)-carbamyl chloride in 1,500 ml. of toluene was refluxed for two hours. The reaction mixture was cooled, filtered, and the filter cake discarded. The filtrate was evaporated to an oil which solidified upon standing to a crystalline cake. This cake was recrystallized first from *n*-hexane and then from diethyl ether and dried.

Procedure (D).—*Preparation of 1-Heterocyclic-4-diethylcarbamylpiperazines.*—To a suspension of 0.30 mole of sodium bicarbonate in a solution of 0.30 mole of the appropriate 1-heterocyclic piperazine in 200 ml. of benzene was added a solution of 0.30 mole of diethylcarbamyl chloride in 100 ml. of benzene over a period of fifteen minutes with vigorous stirring. During the addition, the temperature of the reaction mixture was kept below 50° by cooling in an ice bath. The reaction mixture was then refluxed for one hour and filtered from the insoluble salts. The filtrate was evaporated to a viscous oil which was purified by distilling twice.

Procedure (E).—*Preparation of 1-Heterocyclic-4-diethylcarbamylpiperazines.*—A suspension of 1.0 mole of sodium bicarbonate in a solution of 0.50 mole of the appropriate halogen-substituted heterocycle and 0.50 mole of 1-diethylcarbamylpiperazine in 200 ml. of *o*-dichlorobenzene was refluxed for eight to ten hours. The product was isolated as the free base and purified by distillation.

The properties and analyses of the 1-heterocyclic-4-carbamyl- and -thiocarbamylpiperazines are shown in Table II. In this table is also indicated the general procedure by which each compound was prepared and the yield obtained.

TABLE II.—PROPERTIES AND ANALYSES OF SOME 1-HETEROCYCLIC-4-CARBAMYL- AND -THIOCARBAMYLPIPERAZINES



No.	Het	X	R	Aliphatic	Empirical Formula	Procedure	Yield, %	M. P., ° C.	B. P., ° C.	Analysis for Carbon and Hydrogen Calcd.	Analysis for Carbon and Hydrogen Found	Analysis for Nitrogen Calcd.	Analysis for Nitrogen Found	Analysis for Sulfur Calcd.	Analysis for Sulfur Found	Reference
I	2-Pyridyl	S	H	Ethyl	$C_{12}H_{18}N_4S$	A	40	98–99		C, 57.6 H, 7.20	C, 57.8 H, 7.35	22.4	22.6	12.8	13.0	
II	2-Pyridyl	S	H	Allyl	$C_{13}H_{18}N_4S$	A	38	88–89		C, 59.5 H, 6.87	C, 59.7 H, 6.78	21.4	21.3	12.2	12.2	
III	2-Pyridyl	O	Ethyl	Ethyl	$C_{14}H_{22}N_4O$	E[a]	80	...	165–166 at 0.15 mm.	C, 64.1 H, 8.45	C, 63.8 H, 8.60	21.4	21.1			
IV	2-Pyridyl	O	H	*n*-Hexyl	$C_{16}H_{26}N_4O$	B	34	106.5–107.5		C, 66.2 H, 9.03	C, 66.0 H, 8.97	19.3	19.4			
V	2-Pyridyl	O	H	Cyclohexyl	$C_{16}H_{24}N_4O$	B	52	138.5–139.5		C, 66.7 H, 8.39	C, 66.7 H, 8.26	19.4	19.2			(8)
VI	2-Pyridyl	O	Phenyl	Ethyl	$C_{18}H_{22}N_4O$	C	20	76.0–77.0		C, 69.7 H, 7.14	C, 69.6 H, 7.14	18.1	17.8			
VII	2-Pyrimidyl	S	H	Ethyl	$C_{11}H_{17}N_5S$	A	58	134–135		C, 52.6 H, 6.77	C, 52.9 H, 7.01	27.9	28.0	12.7	12.6	
VIII	2-Pyrimidyl	S	H	Allyl	$C_{12}H_{17}N_5S$	A	59	114–115		C, 54.8 H, 6.46	C, 54.6 H, 6.49	26.6	26.5	12.2	12.1	
IX	2-Pyrimidyl	O	Ethyl	Ethyl	$C_{14}H_{21}N_5O$	D	40	...	172–175 at 2.0 mm.	C, 59.3 H, 8.00	C, 59.4 H, 7.89	26.6	26.6			
X	2-Pyrimidyl	O	H	*n*-Hexyl	$C_{16}H_{29}N_5O$	B	50	129–130		C, 61.9 H, 8.65	C, 61.9 H, 8.82	24.0	23.9			
XI	2-Pyrimidyl	O	H	Cyclohexyl	$C_{16}H_{27}N_5O$	B	57	167–168		C, 62.3 H, 8.02	C, 62.4 H, 7.80	24.2	24.2			(8)
XII	2-Pyrimidyl	O	Phenyl	Ethyl	$C_{17}H_{21}N_5O$	C	20	82.5–83.0		C, 65.7 H, 6.80	C, 65.5 H, 6.58	22.5	22.4			
XIII	5-Chloro-2-pyrimidyl	O	Ethyl	Ethyl	$C_{14}H_{20}ClN_5O$[b]	E[c]	98	76.8–77.6		C, 52.4 H, 6.77	C, 52.4 H, 6.82	23.5	23.7			(3)
XIV	2-Pyrazinyl	S	H	Ethyl	$C_{11}H_{17}N_5S$	A	50	147–148		C, 52.6 H, 6.77	C, 52.8 H, 6.82	27.9	27.9	12.7	12.8	
XV	2-Pyrazinyl	S	H	Allyl	$C_{12}H_{17}N_5S$	A	54	102–103		C, 54.8 H, 6.46	C, 55.1 H, 6.63	26.6	26.4	12.2	12.2	
XVI	2-Pyrazinyl	O	Ethyl	Ethyl	$C_{12}H_{21}N_5O$	E[d]	89	...	156–160 at 0.11 mm.	C, 59.3 H, 8.04	C, 59.3 H, 7.99	26.6	26.3			(3)
XVII	2-Pyrazinyl	O	H	*n*-Hexyl	$C_{14}H_{29}N_5O$	B	22	103.5–104.5		C, 61.9 H, 8.65	C, 62.0 H, 8.62	24.0	23.9			
XVIII	2-Pyrazinyl	O	H	Cyclohexyl	$C_{14}H_{27}N_5O$	B	65	170–171		C, 62.3 H, 8.02	C, 62.4 H, 8.09	24.2	24.3			(8)
XIX	2-Thiazolyl	S	H	Ethyl	$C_{10}H_{18}N_4S_2$	A	43	149–150		C, 46.8 H, 6.25	C, 46.7 H, 6.41	21.9	21.7	25.0	25.2	
XX	2-Thiazolyl	S	H	Allyl	$C_{11}H_{18}N_4S_2$	A	70	133–134		C, 49.2 H, 5.97	C, 49.1 H, 6.12	20.9	20.9	23.9	24.2	
XXI	2-Thiazolyl	O	Ethyl	Ethyl	$C_{12}H_{20}N_4OS$	D	38	...	177–180 at 2.0 mm.	C, 53.7 H, 7.52	C, 53.4 H, 7.67	20.9	21.1	11.9	11.9	
XXII	2-Thiazolyl	O	H	*n*-Hexyl	$C_{14}H_{24}N_4OS$	B	45	124–125		C, 56.8 H, 8.16	C, 56.8 H, 8.34	18.9	18.8	10.8	11.0	(9)
XXIII	2-Thiazolyl	O	H	Cyclohexyl	$C_{14}H_{22}N_4OS$	B	75	145–146		C, 57.1 H, 7.53	C, 57.2 H, 7.75	19.0	18.9	10.9	10.7	(9)
XXIV	2-Thiazolyl	O	Phenyl	Ethyl	$C_{16}H_{20}N_4OS$	C	17	80.5–81.0		C, 60.7 H, 6.37	C, 60.8 H, 6.31	17.7	17.5	10.1	10.2	

[a] Prepared from 2-bromopyridine. [b] Analysis for chlorine: Calcd., 11.9; Found, 11.9. [c] Prepared from 2,5-dichloropyrimidine in ethanol. [d] Prepared from 2-chloropyrazine.

SUMMARY

1. Twenty-four 1-heterocyclic-4-carbamyl- and -thiocarbamylpiperazines have been prepared and described. Five of these compounds received maximum anticonvulsant rating by the general testing methods employed.

2. All of these compounds except three (III, XIII, and XVI) were prepared by the reaction of 1-heterocyclic piperazines, described in paper II (4) of this series, with the appropriate isocyanate, isothiocyanate, or substituted carbamyl chloride.

3. 1-(2-Pyridyl)-, 1-(5-chloro-2-pyrimidyl)-, and, 1-(2-pyrazinyl)-4-diethylcarbamylpiperazines (III, XIII, and XVI) were obtained from the reaction of 1-diethylcarbamylpiperazine with the appropriate halogen-substituted heterocycle.

REFERENCES

(1) Denton, J. J., and Howard, K. L., U. S. pat. 2,459,367.
(2) Hultquist, M. E., and Howard, K. L., U. S. pat. 2,562,036.
(3) Stewart, H. W., U. S. pat. 2,519,715.
(4) Howard, K. L., Stewart, H. W., Conroy, E. A., and Denton, J. J., *J. Org. Chem.*, **18**, 1484(1953).
(5) Conroy, E. A., and Denton, J. J., *ibid.*, **18**, 1489 (1953).
(6) Toman, J. E. P., Swinyard, E. A., and Goodman, L. S., *J. Neurophysiol.*, **9**, 231(1946).
(7) Merritt, H. H., and Putnam, T. J., *Arch. Neurol. Psychiat.*, **39**, 1003(1938).
(8) Stewart, H. W., U. S. pat. 2,602,792.
(9) Stewart, H. W., U. S. pat. 2,602,796.

Piperazines*

V. 1-Heterocyclic-4-monoarylcarbamyl- and -monoarylthiocarbamyl-piperazines

By K. L. HOWARD,† E. A. CONROY, H. W. STEWART, and J. J. DENTON

The preparation and anticonvulsant activity of forty-four new 1-heterocyclic-4-monoarylcarbamyl- and -monoarylthiocarbamylpiperazines are presented. All were prepared by the reaction of 1-heterocyclic piperazines with the appropriate aryl isocyanate or isothiocyanate. Two of these compounds received maximum anticonvulsant rating by the general testing method employed; and one of these, 1-(2-thiazolyl)-4-phenylcarbamylpiperazine, was outstanding in a differential test.

IN A PRECEDING PAPER (1) we have shown that certain 1-heterocyclic-4-monoalkylcarbamyl- and -monoalkylthiocarbamylpiperazines had pronounced anticonvulsant activity when tested against audiogenic seizures in rats. Because of this interesting biological action, the 1-heterocyclic-4-monoarylcarbamyl- and -monoarylthiocarbamylpiperazines listed in Table I were synthesized for similar evaluation.

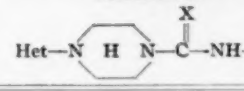
All of the compounds listed in Table I were tested in susceptible rats by the audiogenic-Metrazol® method for the purpose of detecting general anticonvulsant activity. Compounds which exhibited interesting activity in this general testing procedure were tested further by the electroshock method of Merritt and Putnam adapted to the rat and by the intravenous-Metrazol® method in the mouse (2). For a discussion of the audiogenic-Metrazol® and electroshock methods and activity ratings, see paper IV (1) of this series.

In the intravenous-Metrazol® method the activity rating of a compound is based on the increase in the amount of Metrazol® required to produce each successive symptom leading to a final convulsion in mice treated with the compound over that required for an untreated animal. The maximum rating, indicated by plus signs, which a compound may receive by this method is +++++.

Two of the compounds (XXXII and XXXV) in Table I were given maximum activity ratings in the audiogenic-Metrazol® testing procedure;

* Received January 5, 1954, from the Research Department, Calco Chemical Division, American Cyanamid Company, Bound Brook, N. J.
† Present address: Wallerstein Laboratories, 180 Madison Avenue, New York, N. Y.
The authors wish to express their appreciation to R. W. Cunningham, B. K. Harned, and Frank Smith of the Lederle Laboratories Division, American Cyanamid Company, for the pharmacological testing.
The microchemical analyses were carried out by O. E. Sundberg, M. E. Nielsen, and I. H. Prokul.

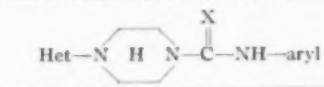
TABLE I.—ANTICONVULSANT ACTIVITY OF SOME 1-HETEROCYCLIC-4-CARBAMYL- AND -THIOCARBAMYL-PIPERAZINES



No.	Het	X	Aryl	Activity[a] mg./Kg. Body Weight: Audiogenic Metrazol®	Intravenous-Metrazol®	Electro-shock
I	2-Pyridyl	S	Phenyl	+++ 500 oral	0 700 oral	0 600 oral
II	2-Pyridyl	O	Phenyl	++i 400 i. p.	0 825 i. p.	0 400 i. p.
III	2-Pyridyl	O	α-Naphthyl	0 500 oral		
IV	2-Pyridyl	O	β-Naphthyl	++i 500 oral	+ 1,000 oral	
V	2-Pyridyl	O	o-Chlorophenyl	+++i 500 oral	0 1,000 oral	± 650 oral
VI	2-Pyridyl	O	o-Ethoxyphenyl	0 500 oral		
VII	2-Pyridyl	O	2-Biphenylyl	++i 500 oral		
VIII	2-Pyridyl	O	o-Nitrophenyl	0 500 oral		
IX	2-Pyridyl	O	m-Chlorophenyl	++i 500 oral	0 1,000 oral	
X	2-Pyridyl	O	m-Nitrophenyl	++ 500 oral		
XI	2-Pyridyl	O	p-Bromophenyl	++i 500 oral	0 1,000 oral	
XII	2-Pyridyl	O	p-Ethoxyphenyl	++i 750 oral	+ 800 oral	
XIII	2-Pyridyl	O	p-Tolyl	0 500 oral	0 1,000 oral	
XIV	2-Pyridyl	O	2,5-Dichlorophenyl	0 500 oral		
XV	2-Pyrimidyl	S	Phenyl	+++ 450 i. p.	0 1,000 i. p.	
XVI	2-Pyrimidyl	O	Phenyl	0i 300 i. p.		0 350 i. p.
XVII	2-Pyrimidyl	O	α-Naphthyl	0 500 oral		
XVIII	2-Pyrimidyl	O	β-Naphthyl	0 500 oral		
XIX	2-Pyrimidyl	O	o-Chlorophenyl	+++i 150 i. p.	+ 325 i. p.	0 150 i. p.
XX	2-Pyrimidyl	O	o-Ethoxyphenyl	0 500 oral		
XXI	2-Pyrimidyl	O	2-Biphenylyl	0i 500 oral		
XXII	2-Pyrimidyl	O	o-Nitrophenyl	0 750 oral		
XXIII	2-Pyrimidyl	O	m-Chlorophenyl	+++i 500 oral	++ 1,000 oral	0 500 oral
XXIV	2-Pyrimidyl	O	m-Nitrophenyl	0 750 oral		
XXV	2-Pyrimidyl	O	p-Bromophenyl	+i 500 oral		
XXVI	2-Pyrimidyl	O	p-Ethoxyphenyl	++i 500 oral	+ 1,000 oral	
XXVII	2-Pyrimidyl	O	p-Tolyl	+i 500 oral	0 1,000 oral	
XXVIII	2-Pyrimidyl	O	2,5-Dichlorophenyl	0 500 oral		
XXIX	2-Pyrazinyl	S	Phenyl	++ 500 oral		0 750 oral
XXX	2-Pyrazinyl	O	Phenyl	++i 150 i. p.	+ 200 i. p.	0 110 i. p.
XXXI	2-Thiazolyl	S	Phenyl	0 500 i. p.		
XXXII	2-Thiazolyl	O	Phenyl	++++ 300 oral ++++ 150 i. p.	++++ 300 i. p.	0 200 i. p.
XXXIII	2-Thiazolyl	O	α-Naphthyl	0 500 oral		
XXXIV	2-Thiazolyl	O	β-Naphthyl	0 1,000 oral		
XXXV	2-Thiazolyl	O	o-Chlorophenyl	++++ 650 oral +++i 170 i. p.	+++ 350 i. p.	0 175 i. p.
XXXVI	2-Thiazolyl	O	o-Ethoxyphenyl	0 500 oral		
XXXVII	2-Thiazolyl	O	2-Biphenylyl	0i 500 oral		
XXXVIII	2-Thiazolyl	O	o-Nitrophenyl	0 500 oral		
XXXIX	2-Thiazolyl	O	m-Chlorophenyl	+++ 750 oral	++ 1,000 oral	0 750 oral
XL	2-Thiazolyl	O	m-Nitrophenyl	++i 500 oral		
XLI	2-Thiazolyl	O	p-Bromophenyl	+++i 500 oral	0 1,000 oral	0 500 oral
XLII	2-Thiazolyl	O	p-Ethoxyphenyl	++ 500 oral	+ 1,000 oral	0 750 oral
XLIII	2-Thiazolyl	O	p-Tolyl	+i 500 oral	+ 1,000 oral	0 750 oral
XLIV	2-Thiazolyl	O	2,5-Dichlorophenyl	0 500 oral		

[a] Significance of ratings: The compounds are tested intraperitoneally (i. p.) or orally (oral) at about the highest dose which does not produce symptoms of toxicity. A rating of +++i indicates an activity of between +++ and ++++.

TABLE II.—PROPERTIES AND ANALYSES OF SOME 1-HETEROCYCLIC-4-CARBAMYL- AND -THIOCARBAMYLPIPERAZINES



No.	Het	X	Aryl	Empirical Formula	Yield, %	M. P., °C.	Analysis for Carbon and Hydrogen Calcd.	Found	Analysis for Nitrogen Calcd.	Found	Analysis for Sulfur Calcd.	Found	Analysis for Chlorine Calcd.	Found	Reference
I	2-Pyridyl	S	Phenyl	$C_{16}H_{18}N_4S$	30	143–141	C, 64.4 H, 6.04	C, 64.6 H, 6.04	18.8	18.9	10.7	10.8			
II	2-Pyridyl	O	Phenyl	$C_{16}H_{18}N_4O$	68	163–163.6	C, 68.1 H, 6.43	C, 67.8 H, 6.68	19.9	19.6					
III	2-Pyridyl	O	α-Naphthyl	$C_{20}H_{20}N_4O$	46	198.0–199.5	C, 72.3 H, 6.06	C, 72.2 H, 6.21	16.8	16.8					
IV	2-Pyridyl	O	β-Naphthyl	$C_{20}H_{20}N_4O$	20	189.5–191.0	C, 72.3 H, 6.06	C, 72.2 H, 6.15	16.8	16.8					(3)
V	2-Pyridyl	O	o-Chlorophenyl	$C_{16}H_{17}ClN_4O$	62	131.5–133.0	C, 60.7 H, 5.41	C, 60.6 H, 5.56	17.7	17.7			11.2	11.4	(3)
VI	2-Pyridyl	O	o-Ethoxyphenyl	$C_{18}H_{22}N_4O_2$	20	99.5–103.0	C, 66.3 H, 6.80	C, 66.3 H, 6.68	17.2	17.2					
VII	2-Pyridyl	O	2-Biphenylyl	$C_{22}H_{22}N_4O$	26	141.0–142.5	C, 73.7 H, 6.18	C, 73.6 H, 6.24	15.6	15.6					
VIII	2-Pyridyl	O	o-Nitrophenyl	$C_{16}H_{17}N_5O_3$	23	121.5–122.5	C, 58.7 H, 5.24	C, 58.6 H, 5.11	21.4	21.4					
IX	2-Pyridyl	O	m-Chlorophenyl	$C_{16}H_{17}ClN_4O$	52	153.0–154.0	C, 60.7 H, 5.41	C, 60.6 H, 5.56	17.7	17.5			11.2	11.4	(3)
X	2-Pyridyl	O	m-Nitrophenyl	$C_{16}H_{17}N_5O_3$	60	159.0–159.5	C, 58.7 H, 5.24	C, 58.5 H, 5.10	21.4	21.5					
XI	2-Pyridyl	O	p-Bromophenyl	$C_{16}H_{17}BrN_4O$[a]	45	183.0–184.0	C, 53.2 H, 4.75	C, 52.8 H, 4.95	15.5	15.1					
XII	2-Pyridyl	O	p-Ethoxyphenyl	$C_{18}H_{22}N_4O_2$	10	183.0–184.5	C, 66.3 H, 6.80	C, 66.5 H, 6.82	17.2	17.0					(3)
XIII	2-Pyridyl	O	p-Tolyl	$C_{17}H_{20}N_4O$	40	188.5–190.0	C, 68.9 H, 6.80	C, 69.1 H, 6.86	18.9	19.0					
XIV	2-Pyridyl	O	2,5-Dichlorophenyl	$C_{16}H_{16}Cl_2N_4O$	45	143.5–144.5	C, 54.7 H, 4.59	C, 54.9 H, 4.45	16.0	15.9			20.2	20.3	
XV	2-Pyrimidyl	S	Phenyl	$C_{15}H_{17}N_5S$	42	157–158	C, 60.2 H, 5.69	C, 59.9 H, 5.58	23.4	23.4	10.7	10.7			
XVI	2-Pyrimidyl	O	Phenyl	$C_{15}H_{17}N_5O$	77	177–178	C, 63.7 H, 6.04	C, 63.8 H, 6.26	24.7	25.0					
XVII	2-Pyrimidyl	O	α-Naphthyl	$C_{19}H_{19}N_5O$	56	208.0–209.0	C, 68.5 H, 5.75	C, 68.4 H, 5.67	21.0	21.2					(3)
XVIII	2-Pyrimidyl	O	β-Naphthyl	$C_{19}H_{19}N_5O$	10	163.0–164.5	C, 68.5 H, 5.75	C, 68.4 H, 5.96	21.0	21.1					
XIX	2-Pyrimidyl	O	o-Chlorophenyl	$C_{15}H_{16}ClN_5O$	58	122.0–123.0	C, 56.7 H, 5.08	C, 56.8 H, 5.23	22.0	21.8			11.2	11.3	(3)
XX	2-Pyrimidyl	O	o-Ethoxyphenyl	$C_{17}H_{21}N_5O_2$	20	96.0–96.5	C, 62.4 H, 6.47	C, 62.2 H, 6.32	21.4	21.4					
XXI	2-Pyrimidyl	O	2-Biphenylyl	$C_{21}H_{21}N_5O$	38	166.0–167.5	C, 70.2 H, 5.89	C, 70.2 H, 5.80	19.5	19.4					
XXII	2-Pyrimidyl	O	o-Nitrophenyl	$C_{15}H_{16}N_6O_3$	43	154.0–154.5	C, 54.9 H, 4.92	C, 54.9 H, 5.00	25.6	25.7					
XXIII	2-Pyrimidyl	O	m-Chlorophenyl	$C_{15}H_{16}ClN_5O$	56	173.5–174.0	C, 56.7 H, 5.08	C, 56.8 H, 4.92	22.0	22.1			11.2	11.4	(3)
XXIV	2-Pyrimidyl	O	m-Nitrophenyl	$C_{15}H_{16}N_6O_3$	74	194.5–196.0	C, 54.9 H, 4.92	C, 55.0 H, 4.97	25.6	25.4					
XXV	2-Pyrimidyl	O	p-Bromophenyl	$C_{15}H_{16}BrN_5O$[b]	43	198.5–199.5	C, 49.7 H, 4.45	C, 49.4 H, 4.54	19.3	19.2					(3)
XXVI	2-Pyrimidyl	O	p-Ethoxyphenyl	$C_{17}H_{21}N_5O_2$	82	170.0–171.5	C, 62.4 H, 6.47	C, 62.3 H, 6.65	21.4	21.2					
XXVII	2-Pyrimidyl	O	p-Tolyl	$C_{16}H_{19}N_5O$	68	175.5–176.5	C, 64.7 H, 6.45	C, 64.7 H, 6.59	23.5	23.5					(3)
XXVIII	2-Pyrimidyl	O	2,5-Dichlorophenyl	$C_{15}H_{15}Cl_2N_5O$	51	148.0–149.0	C, 51.2 H, 4.29	C, 51.4 H, 4.29	19.9	19.9			20.1	20.0	
XXIX	2-Pyrazinyl	S	Phenyl	$C_{15}H_{17}N_5S$	27	173–174	C, 60.2 H, 5.69	C, 60.2 H, 5.85	23.4	23.4	10.7	10.7			

XXX	2-Pyrazinyl	O	Phenyl	$C_{11}H_{12}N_4O$	65	145–146	C, 63.7 H, 6.04	C, 63.6 H, 5.89	24.7	24.9					(3)
XXXI	2-Thiazolyl	S	Phenyl	$C_{11}H_{11}N_3S_2$	23	180–181	C, 55.3 H, 5.27	C, 55.5 H, 5.48	18.4	18.5	21.1	21.0			
XXXII	2-Thiazolyl	O	Phenyl	$C_{11}H_{11}N_4OS$	51	159.5–160.5	C, 58.3 H, 5.58	C, 58.4 H, 5.43	19.4	19.3	11.1	11.0			(4)
XXXIII	2-Thiazolyl	O	α-Naphthyl	$C_{15}H_{13}N_4OS$	70	204.0–205.0	C, 63.9 H, 5.36	C, 64.1 H, 5.56	16.5	16.4	9.45	9.21			(4)
XXXIV	2-Thiazolyl	O	β-Naphthyl	$C_{15}H_{13}N_4OS$	40	186–187	C, 63.9 H, 5.36	C, 64.0 H, 5.32	16.5	16.3	9.45	9.48			(4)
XXXV	2-Thiazolyl	O	o-Chlorophenyl	$C_{11}H_{10}ClN_4OS$	62	117.5–118.5	C, 52.1 H, 4.68	C, 52.2 H, 4.66	17.3	17.2	9.93	9.87	11.0	11.1	(4)
XXXVI	2-Thiazolyl	O	o-Ethoxyphenyl	$C_{13}H_{16}N_4O_2S$	35	94.5–95.0	C, 57.8 H, 6.06	C, 57.9 H, 6.13	16.8	16.8	9.64	9.51			
XXXVII	2-Thiazolyl	O	2-Biphenylyl	$C_{17}H_{15}N_4OS$	46	173.5–175.0	C, 65.9 H, 5.53	C, 65.9 H, 5.69	15.4	15.5	8.79	8.88			
XXXVIII	2-Thiazolyl	O	o-Nitrophenyl	$C_{11}H_{10}N_5O_3S$	19	125.0–126.0	C, 50.5 H, 4.53	C, 50.5 H, 4.57	21.0	21.0	9.62	9.56			
XXXIX	2-Thiazolyl	O	m-Chlorophenyl	$C_{11}H_{10}ClN_4OS$	27	146.5–147.5	C, 52.1 H, 4.68	C, 52.0 H, 4.63	17.3	17.3	9.93	9.88	11.0	10.8	(4)
XL	2-Thiazolyl	O	m-Nitrophenyl	$C_{11}H_{10}N_5O_3S$	15	178.5–180.0	C, 50.5 H, 4.53	C, 50.2 H, 4.44	21.0	20.8	9.62	9.41			
XLI	2-Thiazolyl	O	p-Bromophenyl	$C_{11}H_{10}BrN_4OS^c$	54	172.5–173.5	C, 45.8 H, 4.12	C, 46.0 H, 4.32	15.3	15.3	8.72	8.63			(4)
XLII	2-Thiazolyl	O	p-Ethoxyphenyl	$C_{13}H_{16}N_4O_2S$	48	188.0–190.0	C, 57.8 H, 6.06	C, 57.7 H, 5.86	16.8	16.7	9.64	9.53			(4)
XLIII	2-Thiazolyl	O	p-Tolyl	$C_{12}H_{14}N_4OS$	73	177.0–178.5	C, 59.6 H, 6.00	C, 59.4 H, 6.11	18.5	18.5	10.6	10.8			(4)
XLIV	2-Thiazolyl	O	2,5-Dichlorophenyl	$C_{11}H_9Cl_2N_4OS$	27	169.0–170.0	C, 47.1 H, 3.95	C, 47.1 H, 3.87	15.7	15.7	8.97	8.78	19.9	19.9	

[a] Analysis for bromine: Calcd., 23.1; Found, 22.4. [b] Analysis for bromine: Calcd., 22.0; Found, 21.8. [c] Analysis for bromine: Calcd., 21.8; Found, 22.0.

while neither of these showed activity at the dose tested in the electroshock method, they did receive the highest activity ratings of those compounds in Table I which were tested by the intravenous-Metrazol® method. The most outstanding compound was 1-(2-thiazolyl)-4-phenylcarbamylpiperazine (XXXII).

EXPERIMENTAL

General Procedure.—To a stirred solution of the appropriate 1-heterocyclic piperazine in diethyl ether was added a solution of an equivalent amount of the appropriate aryl isocyanate or isothiocyanate in diethyl ether over a period of ten minutes at room temperature. During the addition the reaction mixture was cooled in an ice bath, if necessary. The precipitate which formed was removed by filtration, recrystallized from an organic solvent, and dried.

Petroleum ether was used as the reaction medium in the case of compound II, and boiling pyridine in the case of compounds IV, XVIII, and XXXIV. It was necessary to dilute the pyridine solution with water in order to precipitate the products.

The properties and analyses of the 1-heterocyclic-4-monoarylcarbamyl- and -monoarylthiocarbamylpiperazines are shown in Table II.

SUMMARY

1. Forty-four 1-heterocyclic-4-monoarylcarbamyl- and -monoarylthiocarbamylpiperazines have been prepared, described, and tested as anticonvulsants. Two of these compounds received maximum anticonvulsant rating by the general testing method employed; and one of these, 1-(2-thiazolyl)-4-phenylcarbamylpiperazine, was outstanding in a differential test.

2. All of these compounds were prepared by the reaction of 1-heterocyclic piperazines, described in paper II (5) of this series, with the appropriate aryl isocyanate or isothiocyanate.

REFERENCES

(1) Conroy, E. A., Quinones, N. Q., Howard, K. L., Stewart, H. W., and Denton, J. J., THIS JOURNAL, **43**, 625 (1954).
(2) Orloff, M. J., Williams, H. L., and Pfeiffer, C. C., *Proc. Soc. Exptl. Biol. Med.*, **70**, 254(1949).
(3) Stewart, H. W., U. S. pat. 2,602,792.
(4) Stewart, H. W., U. S. pat. 2,602,796.
(5) Howard, K. L., Stewart, H. W., Conroy, E. A., and Denton, J. J., *J. Org. Chem.*, **18**, 1484(1953).

Isotonic Solutions*

III. Amino Acids and Sugars

By THADDEUS S. GROSICKI† and WILLIAM J. HUSA‡

The commonly used methods of calculating isotonic solutions are based on assumptions which often give erroneous results. The present study deals with the hemolytic method using rabbit blood and human blood. Direct determinations were made of the degree of hemolysis occurring in solutions of various amino acids and sugars as compared with the degree of hemolysis in solutions of sodium chloride.

THE HEMOLYTIC METHOD of studying the permeability of red blood cells was introduced by Hamburger (1) in 1883 and was also used by Grijns (2), Hedin (3) and other investigators whose work has been thoroughly reviewed by Davson and Danielli (4) and by Ponder (5).

Wokes (6) and Husa and Adams (7) applied the hemolytic method to the study of isotonic solutions for parenteral use. Wokes determined the ratio isotonic concentration/hemolytic concentration for a number of substances and stated that, knowing this ratio, it is possible to estimate the isotonic concentration of an unknown solution by diluting the latter until it just produced hemolysis. A solution diluted with half its volume of distilled water was not considered to be isotonic if it produced hemolysis. Husa and Adams studied the effect of various substances on hemolysis of human red blood corpuscles and found that a number of these failed to prevent hemolysis when used alone in aqueous solutions of a concentration isosmotic with the blood. It was established, however, that in the presence of appreciable concentrations of sodium chloride, some of the substances were able to contribute slightly to the prevention of hemolysis. These investigators stressed the importance of making hemolytic tests, particularly as a check on freezing point data for the preparation of parenteral solutions.

In the present investigation the hemolysis of rabbit and human erythrocytes in solutions of substances of varying concentration was determined quantitatively and the data thus obtained were used in calculating the value of *i* (isotonic coefficient) by the osmotic factor method.

TABLE I.—VALUES OF i USING RABBIT ERYTHROCYTES

Amino Acids	At 25% Hemolysis	At 50% Hemolysis	At 75% Hemolysis	75 Average
Glycine	1.10	1.13	1.18	1.14
β-Alanine	1.17	1.19	1.25	1.20
dl-Alanine	1.19	1.20	1.23	1.21
dl-Isoleucine	1.16	1.18	1.21	1.18
Sugars				
Dextrose	1.20	1.20	1.24	1.21
Sucrose	1.17	1.19	1.24	1.20
Lactose	1.22	1.24	1.27	1.24
Salts				
Sodium Acetate	1.80	1.78	1.78	1.79
Sodium Nitrate	1.93	1.93	1.97	1.94
	(In presence of 0.2% NaCl)			
Amino Acids				
Glycine	0.95	0.94	0.95	0.95
β-Alanine	0.96	0.96	0.97	0.96
dl-Alanine	0.94	0.95	0.95	0.95
Sugars				
Dextrose	1.13	1.15	1.16	1.15
Sucrose	1.21	1.23	1.27	1.24
Lactose	1.19	1.21	1.26	1.22
	(In presence of 0.3% NaCl)			
Dextrose	1.19	1.20	1.18	1.19

EXPERIMENTAL

Source of Blood.—The rabbit blood used in the experimental work was obtained by heart puncture. The blood was immediately defibrinated by gentle rotation in a flask with glass beads until the fibrin had separated. The defibrinated blood was then poured into a small flask and aerated by gently swirling the flask for approximately five minutes. The human blood used in the course of the experiments was obtained from the veins of the left arm of a human male, age thirty years, by means of a hypodermic syringe. It was treated in exactly the same manner as the rabbit blood. Each experiment was repeated using a fresh sample of rabbit or human blood.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in salt solutions of varying concentration was essentially that of Hunter (8). It is based on the fact that the oxyhemoglobin liberated by a hypotonic solution is a direct function

* Received January 28, 1954, from the College of Pharmacy, University of Florida, Gainesville.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
This paper is based on a dissertation presented to the Graduate Council of the University of Florida by Thaddeus S. Grosicki in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Present address: School of Pharmacy, University of Arkansas, Little Rock.
‡ Head, Department of Pharmacy, University of Florida, Gainesville.

of the number of cells hemolyzed. Furthermore, as complete hemolysis results from laking of erythrocytes in a 0.10 per cent sodium carbonate solution, it is possible to express the hemolysis occurring in a given strength of salt solution in terms of total hemolysis. A quantitative determination of partial hemolysis in any strength of salt solution can be made by centrifuging off the unhemolyzed cells and determining the oxyhemoglobin in the supernatant fluid by means of a photoelectric colorimeter.

The present method consisted of transferring, by means of a volumetric pipet, 5 cc. of the designated salt solutions into each of two test tubes. With a volumetric pipet 0.05 cc. of fresh defibrinated and aerated blood was then added to each of two test tubes; the tubes were stoppered and inverted several times to obtain complete mixing. The blood-salt mixtures were then placed in a water bath at 25° ± 1° for forty-five minutes. At the end of this period, the mixtures were centrifuged for three minutes at approximately 1000 r. p. m. Readings of the light transmission in the supernatant liquid were made in a Klett-Summerson photoelectric colorimeter with a No. 54 green filter.

Complete hemolysis of rabbit or human blood was obtained by placing 5 cc. of 0.1 per cent sodium carbonate solution in a test tube, adding to it 0.05 cc. of blood and mixing immediately. The reading obtained for the sodium carbonate-blood mixture in the photoelectric colorimeter represented 100 per cent hemolysis. Five separate readings were made for each blood sample. The average of five reading was used in the calculations.

The blank, used to cancel any differences in light absorption due to opalescence or bilirubin, was prepared by placing 0.05 cc. of blood in 5 cc. of 0.85 per cent sodium chloride solution, allowing to stand for forty-five minutes and centrifuging in like manner.

The per cent hemolysis occurring in duplicate tubes of each strength of salt solution was calculated by dividing the reading obtained from each salt concentration by the reading obtained from complete laking with sodium carbonate. These readings multiplied by 100 represent the per cent of total hemolysis in each strength of salt solution.

Variable erythrocyte fragility in solutions of the same concentration with different samples of rabbit and human blood was noted and taken into consideration. Any error due to variable erythrocyte fragility was minimized by determining the degree of hemolysis in varying concentrations of sodium chloride for each blood sample. Thus a direct comparison between the degree of hemolysis occurring in a certain concentration of sodium chloride and some other solution was possible. With samples of blood obtained from different rabbits the degree of hemolysis in the same concentration of sodium chloride varied from less than one per cent to as much as fifty per cent. In the case of human blood, which was obtained from the same individual, the variations were much smaller. It should be noted, however, that the degree of hemolysis occurring in duplicate tubes of the same sodium chloride concentration for the same blood sample did not vary by more than 3%.

TABLE II.—VALUES OF i USING HUMAN ERYTHROCYTES

Amino Acids	At 25% Hemolysis	At 50% Hemolysis	At 75% Hemolysis	Average
Glycine	1.17	1.22	1.25	1.21
β-Alanine	1.19	1.25	1.28	1.24
dl-Alanine	1.23	1.26	1.30	1.26
Sugars				
Dextrose	1.14	1.17	1.20	1.17
	(In presence of 0.2% NaCl)			
Amino Acids				
Glycine	0.90	0.91	0.90	0.90
β-Alanine	0.94	0.94	0.96	0.95
dl-Alanine	0.97	0.96	0.94	0.96
Sugars				
Dextrose	0.65	0.67	0.67	0.66

Calculating i Value.—Hunter (8) has shown that if the per cent hemolysis is plotted against the strength of salt solution, the curve obtained will ordinarily be sigmoid in character. On rectangular coordinate paper the salt solutions in order of decreasing strength were marked off along the abscissa, and the per cent hemolysis in increasing order along the ordinate. Typical sigmoid curves were obtained for each blood sample. At least seven sodium chloride concentrations ranging from 0.36 to 0.54% (0.36, 0.40, 0.42, 0.44, 0.46, 0.48, 0.54) were used. With rabbit erythrocytes little or no hemolysis occurred in the 0.54 per cent salt solution and hemolysis was usually complete in the 0.36 per cent salt solution.

If we know the concentrations of sodium chloride and any other compound giving the same degree of hemolysis we can calculate the value of i (isotonic coefficient) for the other compound according to the following equation:

$$\frac{\begin{pmatrix} i \text{ value} \\ \text{for NaCl} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 cc.} \\ \text{of solution} \end{pmatrix}}{\text{Gm.-molecular weight of NaCl}} = \frac{\begin{pmatrix} i \text{ value} \\ \text{for other} \\ \text{compound} \end{pmatrix} \begin{pmatrix} \text{Gm. of other} \\ \text{compound in} \\ \text{100 cc. of} \\ \text{solution} \end{pmatrix}}{\text{Gm.-molecular weight of compound}}$$

In using the above equation the value of i for sodium chloride was taken as 1.86, which is the accepted value of i for the 0.9% (0.15 M) sodium chloride solution (9). In more dilute solutions the value of i for sodium chloride increases slightly. For example, for a 0.5% (0.08 M) sodium chloride solution i has a value of approximately 1.87. Therefore, using 1.86 as the value of i for sodium chloride, where its concentration is 0.1 M or less, introduces only a small error.

Curves showing the degree of hemolysis in sodium chloride solutions as well as for the compound being tested were plotted on rectangular coordinate paper for each blood sample. From these curves the concentrations in Gm./100 cc. of sodium chloride and of the compound being tested causing 25, 50, and 75 per cent hemolysis were determined. These values were then substituted in the osmotic factor equation and the value of i for the compound, at concentrations giving 25, 50, and 75% hemolysis was determined. Duplicate samples of each sodium chloride concentration and of the compound being

tested were used and each experiment was repeated using a different blood sample. Each i value in the tables is the average of the results on two different blood samples.

Tests were also made to determine whether the presence of KCl and $CaCl_2$ would alter the i value of dextrose. In presence of 0.18% NaCl and a quantity of KCl equivalent to 0.02% NaCl, the average i value for dextrose was 1.15, using rabbit erythrocytes. In presence of 0.185% NaCl and a mixture of KCl and $CaCl_2$ equivalent to 0.015% NaCl the i value for dextrose was 1.20 using rabbit erythrocytes; the KCl and $CaCl_2$ were present in the same proportion as in Ringer's Solution, U. S. P. XIV.

DISCUSSION

The isotonic coefficient, i, was introduced by van't Hoff to cover all types of deviation from ideal behavior without any question as to their origin (11). The i values determined by the hemolytic method depend partly on the properties of the substance whose i value is being determined and partly on the permeability of the erythrocyte of the particular species to the substance. The use of sodium chloride as a standard of reference eliminated any difficulties of reproducibility of the biological factors involved, particularly variable erythrocyte fragility in different individuals. The variation in i values obtained from the blood of different rabbits was of the order $\pm 2\%$. Even though the human blood was all taken from one person there was a day to day variation in fragility; the use of sodium chloride as a standard of reference enabled the determination of i values with a variation of $\pm 2\%$ when using blood drawn on different days.

In calculations of isotonic solutions the value of i is commonly assumed to be the same as the number of ions into which the compound dissociates. Husa and Rossi (9) have shown that the error involved in this assumption is within allowable limits for practical work, provided the concentration of the solution is 0.1 M or less; it must further be provided that the erythrocytes are not permeable to the compound.

The i value of solutes is sometimes determined from the freezing point or other colligative property of a solution. However, i values obtained entirely by such methods are open to serious question because some solutes will pass through certain membranes; thus a solution which seems to have a proper i value may fail to prevent hemolysis. Solutions having the same osmotic pressure based on measurements of freezing points or other colligative properties are sometimes called "isotonic"; however, the better practice is to designate such solutions as "isosmotic" and to reserve the term "isotonic" for solutions having equal osmotic pressure with reference to a particular plasmatic membrane, e. g., erythrocyte.

Whether the results of hemolytic tests *in vitro* exactly parallel the results that would occur *in vivo* is not definitely known. However, it would appear that solutions which fail to prevent hemolysis *in vitro* should certainly be questioned for use *in vivo*.

Amino Acids.—The i values found for amino acids were approximately 1.20 using rabbit erythrocytes. These values might be interpreted as showing about 20% dissociation of these amino acids but this is contrary to the recognized chemical properties of amino acids which are known to be very weak acids. It is realized that in solutions of some substances, erythrocytes lose electrolytes from within the cell, thus causing a decrease in internal osmotic pressure and a corresponding increase in resistance to hemolysis. It appears that such exosmosis from erythrocytes may occur in solutions of amino acids. As a result the cells are in equilibrium with lower concentrations of amino acids than would otherwise be the case and the i value is higher than can be otherwise explained from the known properties of amino acids.

It has been stated (10) that the abnormal permeability of erythrocytes in solutions of some nonelectrolytes can be corrected in some cases by addition of a small proportion of an electrolyte such as NaCl. The results in Tables I and II support this statement, since for both rabbit and human erychtocytes the presence of 0.2% NaCl reduced the apparent i value of amino acids from about 1.20 to slightly less than 1.00.

Sugars.—Dextrose, sucrose, and lactose gave i values of about 1.20 using rabbit erythrocytes, the results thus being the same as for amino acids. The fact that the i values are greater than 1.00 may be ascribed to loss of electrolytes from within the cell. However, in case of the sugars, the i values in presence of 0.2% NaCl remained about 1.20 using rabbit erythrocytes. In the case of sugars, the presence of 0.2% NaCl apparently does not correct the abnormal exosmosis. The replacement of part of the 0.2% NaCl by KCl, or a mixture of KCl and $CaCl_2$, likewise did not correct the abnormal outward permeability.

Using human erythrocytes, dextrose gave an average i value of 1.17, or practically the same as the value using rabbit erythrocytes. However, using human erythrocytes in presence of 0.2% NaCl dextrose gave an i value of 0.66. It appears that in presence of 0.2% NaCl the human erythrocyte is permeable to dextrose so that the erythrocytes appear to be in equilibrium with a higher concentration of dextrose than would be the case if no penetration had occurred, thus giving an apparently lower i value.

SUMMARY

1. Quantitative data obtained by the hemolytic method enabled the calculation of the isotonic coefficients or i values of some amino acids, sugars and salts.

2. Amino acids and sugars gave i values of about 1.20 using rabbit erythrocytes. The fact that these values exceed 1.00 is apparently due to a change in permeability of the corpuscular membrane, resulting from electrolyte loss from within the cell. The presence of 0.2% NaCl restores normal permeability in case of amino acids but not for sugars.

3. Human blood gave the same results as rabbit blood for amino acids but not for dextrose.

4. In solutions of dextrose alone the *i* value of dextrose using human erythrocytes was 1.17, indicating exosmosis and no apparent permeability to dextrose; in presence of 0.2% NaCl the *i* value dropped to 0.66 showing penetration of the erythrocytes by dextrose.

REFERENCES

(1) Lund, C. G., Peulicke Nielsen, E., and Pedersen-Bjergaard, K., "The Preparation of Solutions Isosmotic With Blood, Tears, and Secretions," Heinemann Medical Books Ltd., London, 1947, p. 16.
(2) Grijns, G., *Pflügers Arch. ges. Physiol.*, **63**, 86(1896); cited by Hober, R., "Physical Chemistry of Cells and Tissues," 1st ed., Blakiston Co., Philadelphia, Pa., 1945, p. 238.
(3) Hedin, S. G., *Pflügers Arch. ges. Physiol.*, **68**, 229 (1897); *ibid.*, **70**, 525(1898); cited by Hober, R., "Physical Chemistry of Cells and Tissues," 1st ed., Blakiston Co., Philadelphia, Pa., 1945, p. 238.
(4) Davson, H., and Danielli, J. F., "The Permeability of Natural Membranes," The Macmillan Co., New York, 1943.
(5) Ponder, E., "Hemolysis and Related Phenomena," Grune & Stratton, New York, 1948.
(6) Wokes, F., *Quart. J. Pharm. Pharmacol.*, **9**, 455 (1936).
(7) Husa, W. J., and Adams, J. R., THIS JOURNAL, **32**, 329(1944).
(8) Hunter, F. T., *J. Clin. Invest.*, **19**, 691(1940).
(9) Husa, W. J., and Rossi, O. A., THIS JOURNAL, **31**, 270(1942).
(10) Hober, R., "Physical Chemistry of Cells and Tissues," 1st ed., Blakiston Co., Philadelphia, Pa., 1945, p. 238.
(11) Glasstone, S., "Textbook of Physical Chemistry," 2nd ed., D. Van Nostrand Company, Inc., New York, 1946, p. 674.

Gallic Acid Ethanolamide (N-(β-Hydroxyethyl)-3,4,5-trihydroxybenzoic Acid Amide)*

By GERHARD JULIUS HAAS† and MARGARET ROSE ZENTNER

The present paper is concerned with the preparation of the ethanolamide of gallic acid and its utility as a solubilizer for riboflavin.

THE COMPOUND was synthesized in two steps from gallic acid (1). In the first step the methyl ester was prepared using either sulfuric acid or the sulfonate type resin Amberlite® IR 120 (3) as esterification catalyst. Then the methyl gallate was reacted with ethanolamine to form gallic acid ethanolamide. This compound was found useful as a solubilizer for riboflavin (2). On shaking at room temperature an excess of riboflavin in 100 cc. of a 1.5% gallic acid ethanolamide solution for thirty-six hours, 106 mg. of riboflavin was dissolved;[1] whereas, the solubility of vitamin B_2 in water alone after shaking for ninety-six hours at room temperature was found to be 12.3 mg. per 100 cc. The compound also proved to be compatible with all the usual constituents of polyvitamin solutions.

* Received March 26, 1954, from the Pharmaceutical Research Laboratories of Hoffmann-La Roche Inc., Roche Park, Nutley, N. J.
† Present address: Liebmann Breweries, Inc., 36 Forrest Street, Brooklyn 6, N. Y.
The authors wish to thank Dr. M. Hoffer for the many valuable suggestions which greatly facilitated the project.
[1] Determined fluorometrically.

EXPERIMENTAL

Preparation of Methyl Gallate.—*Method A.*—Nine hundred and seventy-eight grams of gallic acid (dried at 110° for sixteen hours) was dissolved in 4,300 cc. methanol. When solution was complete 177 Gm. of concentrated sulfuric acid was added and the mixture refluxed for thirty hours. After that time 245 cc. of concentrated ammonia was added (until the solution was slightly alkaline) and about 5,700 cc. of methanol distilled off. The reaction mixture was poured into 1,800 cc. water. Crystallization set in almost at once. The solution was stirred for fifteen minutes and placed in the refrigerator. The next day the methyl gallate was filtered off and redissolved in 2,000 cc. acetone. Six and nine-tenths grams of a water- and acetone-insoluble solid, probably a gallic acid polymer, was removed by filtration; then most of the acetone was distilled off from the filtrate. Hot water (1,500 cc.) was added; this removed the last traces of acetone by evaporation. After leaving the mixture in the refrigerator overnight the methyl gallate was filtered off. Nine hundred and thirty-three grams of product (m. p. 199–201°) resulted. From the combined mother liquors of both crystallizations another 55 Gm. of the same m. p. resulted. Total yield: 988 Gm., 93.5% of theory. The melting point agrees well with those reported in Beilstein (199°, 202°). The analysis of a sample of another run of material of the same melting point yielded:

Anal.—Calcd. for $C_9H_{10}O_5$. Theory C: 52.13. Found 52.46. Theory H: 4.38. Found 4.39. The

yield was about 8% less when the reaction was only allowed to proceed for sixteen hours.

Method B.—Sixty-one and two-tenths grams of dried gallic acid was dissolved in 274 cc. methanol. Twenty-three grams of Amberlite® IR 120 (converted to the acid form by washing three times with 10% sulfuric acid by decantation, then washed neutral with water and dried at 110° for twenty hours) was added and the mixture was refluxed for thirty-two hours. The Amberlite® was filtered off, an equal amount of water was added and the solution passed through a column of Amberlite® IR 4 (3) to remove unreacted gallic acid. The methanol was then evaporated and 56 Gm. of material of m. p. 192–192.5° resulted; the mixed melting point with methyl gallate of m. p. 200–202° was 198–200°.

Preparation of Gallic Acid Ethanolamide.—Fifty grams of methyl gallate and 40.8 Gm. monoethanolamine was heated at 115–120° for eight hours. The reaction mixture which had become solid during that period was dissolved in 1,300 cc. hot water and concentrated hydrochloric acid (35 cc.) was added to *p*H 4. The solution was heated for ten minutes with 2.5 Gm. charcoal (Norite® SV), filtered and concentrated to 310 cc. *in vacuo*. Upon cooling overnight 47 Gm. of m. p. 217.5–218.8° resulted. After recrystallization from water and extraction of the solid with acetone to remove a slight amount of an acetone-soluble impurity, 40 Gm. of a product melting at 219–220.5° (corr.) resulted.

Anal.—Calcd. for $C_9H_{11}O_5N$. Theory C: 50.67. H: 5.20, N: 6.57. Found C: 50.69. H: 5.17. N: 6.75.

Its solubility in water by heating and cooling is 1.5%; by shaking, 1%; in absolute ethanol, 0.5%; in 50% ethanol, 5.4%. The solubility in water is markedly increased by polyhydroxy compounds such as dextrose and ascorbic acid.

When using ethyl gallate instead of methyl gallate the yield was smaller and the material of much lower purity.

Gallic acid ethanolamide shows characteristic color reactions with barium hydroxide by which it may be differentiated from gallic acid and methyl gallate. One drop of a 3.6% barium hydroxide solution added to 5 cc. of 0.01% solutions of the three compounds produces the following color changes:

Gallic acid	A bluish black color and bluish black precipitate.
Methyl gallate	A yellow color and slow precipitation of an orange-yellow precipitate.
Gallic acid ethanolamide	An orange color and no precipitate for four hours.

REFERENCES

(1) Zentner, M. R., and Haas, G. J., U. S. pat. 2,571,325.
(2) Zentner, M. R., U. S. pat. 2,571,326.
(3) Obtained through the courtesy of Rohm & Haas Co.

A Note on the Effects of Feeding Saw Palmetto Berries, Serenoa Repens (Bartram) Small, to Rats*

By SELDON DICK FEURT† and LAURETTA E. FOX‡

The growth of suckling rats was depressed when their dams were fed Saw Palmetto berries as 50 per cent by weight of their diet. Young rats fed a similar diet showed growth depression and developed gangrenous conditions. Adult rats did not lose weight nor develop gangrene when fed a diet containing Saw Palmetto berries for seventy-five days.

SWANTON (1) IN relating the experiences of a group of sailors shipwrecked off the Florida coast in 1699 states that the Ais Indians considered ripe palmetto berries a delicacy, but the sailors compared their taste to "rotten cheese steeped in tobacco juice." Van Doren (2) in *The Travels of Wm. Bartram* states that the fruits while frequently eaten prove purgative in excess. Read (3) reports that bears become quite fat and somnolent during the ripe berry season and are easy prey for hunters. Dixon (4) in a paper given before the Florida State Pharmaceutical Association claims that cows give more and richer milk when eating palmetto berries than any other food. In the light of these reports an investigation of the effects of feeding palmetto berries to rats seemed promising.

Growth Depression in Suckling Rats Whose Dams Were Fed Saw Palmetto Berries.—Individual weights of new-born rats of the Sprague-Dawley-Holtzman strain were recorded. The number of rats in each litter was reduced to six, runts and large pups being destroyed as advised by Daggs (5). The dams of the five control litters received a commercial laboratory ration; the dams of another five litters received the same powdered commercial ration mixed with 50% by weight of ground Saw Palmetto berries (partially dried ripe fruits supplied by S. B. Penick Company). The sucklings were weighed at

* Received September 19, 1953, from the College of Pharmacy, University of Florida, Gainesville.
Abstracted in part from a dissertation submitted to the Graduate School of the University of Florida by Seldon Dick Feurt in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Associate Professor of Pharmacology, School of Pharmacy, University of Georgia, Athens.
‡ Assistant Professor of Pharmacognosy and Pharmacology, College of Pharmacy, University of Florida.
The authors are indebted to S. B. Penick and Company, New York, for the partially dried ripe fruits of Saw Palmetto used in these experiments.

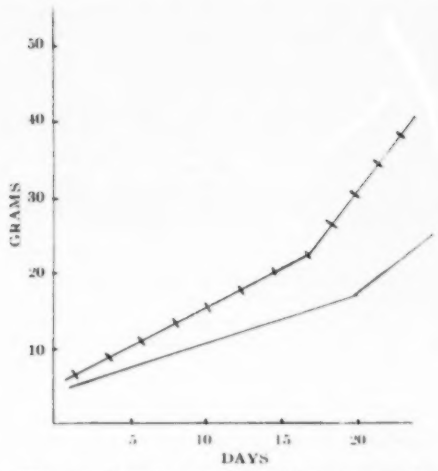


Fig. 1.—Growth rate from birth to weaning. Cross-hatched line indicates growth of control litters. Solid line indicates growth of litters whose dams received palmetto berries.

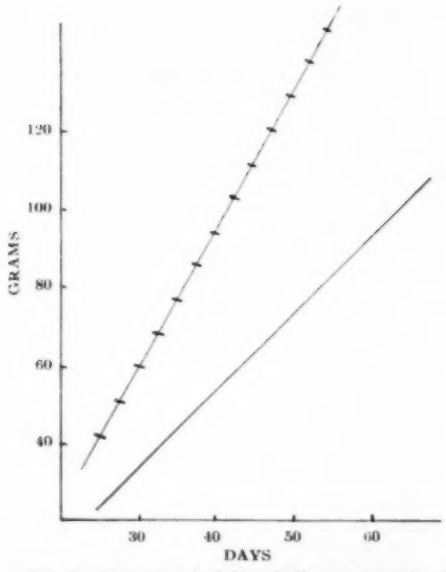


Fig. 2.—Gross-hatched line indicates growth of control rats after weaning. Solid line indicates growth of rats receiving palmetto berries.

five-day intervals until weaned and their average weights recorded as shown in Fig. 1. Analysis of the data revealed a significant difference between the means of the weights of the two groups at both ten and fifteen days of age. The growth of suckling rats was depressed by oral administration of Saw Palmetto berries to their dams. The weights of the dams remained uniform throughout the experiment. Food was in excess at all times and the amount replenished each day was approximately the same for all cages. No purgative action was noted.

Fig. 3.—Gangrenous condition of young rat fed palmetto berries. Note loss of right foreleg, the gangrenous condition of the toes of the forefoot, and the clubbing and loss of phalanges of hind feet.

The results suggest that Saw Palmetto berries decrease the quantity or quality of the dam's milk or that a substance from palmetto which is excreted in the dam's milk retards the growth of sucklings. The growth rate of young rats was used by Daggs (5) to test indirectly for lactogenic action.

Effects of Feeding Saw Palmetto Berries to Young Rats.—The young rats of the previous experiment were weaned and continued on the same diets as those received by their dams until seventy-five days old. Growth depression of the young rats eating Saw Palmetto berries was evident throughout the experiment as shown in Fig. 2.

The young rats receiving Saw Palmetto berries developed vasoconstriction as evidenced by their cold, pale extremities. When the rats were approximately forty days oid gangrenous conditions of the toes and tips of their ears were observed. These gangrenous conditions progressed to the point where whole limbs were lost (Fig. 3). Our findings as to probable causes of these conditions will be reported in a subsequent paper.

The rats were sacrificed when seventy-five days old; their body weights and the weight of the spleen, liver, kidney, testis, ovary and uterus, and seminal vesicles were recorded. There was no significant difference between the weights of these organs for the two groups when the weights were calculated as per cent of body weight. Growth retardation of the young rats eating Saw Palmetto berries was uniform in regard to skeletal and organ development.

Tissue sections were prepared in the customary manner and stained with eosin and hematoxylon, but no changes of pathological significance were observed.

Effects of Feeding Saw Palmetto Berries to Adult Rats.—In a similar feeding experiment groups of 10 adult rats of the same strain and of mixed sex were fed the two diets described above for seventy-five days. No weight depression and no gangrenous conditions were observed in this period. Adults are often more resistant than young.

SUMMARY

1. The growth of suckling rats is depressed by feeding their dams a diet consisting of 50 per cent by weight of Saw Palmetto berries.

2. Young rats fed Saw Palmetto berries manifest growth depression and develop grangrenous conditions of their extremities which progresses to the loss of limbs.

3. Adult rats did not develop gangrenous conditions nor lose weight under the conditions of this experiment.

4. No histological changes of pathological significance were observed in animals fed Saw Palmetto berries.

REFERENCES

(1) Swanton, J. R., "The Early History of the Creek Indians and Their Neighbors," Bureau of American Ethnology, Bull. No. 73, 1922, pp. 384–392.
(2) Van Doren, Mark, "The Travels of Wm. Bartram," Macy-Masius, New York, 1928, p. 81.
(3) Read, J. B., *Am. J. Pharm.*, **Apr. 1879,** p. 169.
(4) Dixon, J. M., "Saw Palmetto," Minutes of the Florida State Pharmaceutical Association Meeting, June 15, 1910.
(5) Daggs, R. G., *J. Nutrition*, **9**, 575(1935).

Book Notices

Les Détersifs en Hygiène et Cosmétique. By W. KOPACZEWSKI. Masson and Cie, Paris, 1954. 143 pp. 16 x 25 cm. Paper bound, uncut. Price *fr.* 1,800.

The author's preface was written at the Hôtel-Dieu, in Paris, where he succumbed after putting the finishing touches to this last volume of his trilogy on the detergents, the first volumes of which referred to the experimental foundations and the pharmaceutical use of detergents. The volume maintains the high scientific standard of its predecessors. The author writes for the specialist to whom he presents not ready-made predigested formulas, but a guide in working out useful nontoxic preparations on the experimental basis. A long and important part of the book is given over to the problem of biological resorptions including sensitization (allergy), shock, and neoplasms. This carefully written section gives the book added importance and usefulness.

Wine as Food and Medicine. By SALVATORE P. LUCIA. The Blakiston Co., Inc., New York, 1954. 149 pp. 14 x 21.5 cm. Price $3.

The author discusses the chemistry of wine, its physiologic effects, its nutritional value, and finally its therapeutic action in diseases affecting various systems and organs. The use of wine in geriatrics and convalescence is also stressed. The author has searched the literature diligently in an effort to support his thesis that wine has its uses in many of the major afflictions known to mankind. This wide application of oenotherapy, based largely on references to the literature of days long gone by, seems to exaggerate its true value even to those who subscribe to his thesis that "in the wild scramble for therapeutic specificity, little thought has been given to the psychotherapeutic effects of the simple adjuvants to living that make life worth while."

II Congreso Luso-Espanhol de Farmácia. 3 vols. Under the auspices of the Faculty of Pharmacy, Porto, Portugal. 1952. Vol. I, 410 pp., Vol. II, 585 pp., Vol. III, 1,642 pp. 26 x 19.5 cm.

The three volumes bear eloquent testimony not only to the industry of the hard-working editorial committee, but also to the importance of the topics discussed and to the value of the papers presented at the Second Portuguese-Spanish Pharmacy Congress held at Porto from May 11–17, 1952. Space does not permit the reviewer to refer individually to any of the hundreds of papers reprinted or abstracted on these more than 2,000 pages.

The subjects covered extend over a wide range including chemistry, toxicology, hydrology, pharmacognosy, pharmacodynamics, microbiology, parasitology, hygiene, galenic and industrial pharmacy, professional problems, history, legislation, and deontology.

Publications of this type, welcome as they are, are not usually indexed in any of the current abstract or indexing journals which raises the question of their usefulness to a wider public.

Antibiotika-Fibel—Indikation und Anwendung der Chemotherapeutika und Antibiotika. By ALBERT M. WALTER and LUDWIG HEILMEYER. Georg Thieme Verlag, Stuttgart, 1954. (In the U. S. and Canada: Intercontinental Medical Book Corporation, New York.) 813 pp. 16 x 21 cm. Price *DM* 79 ($18.80).

The title of this book is thoroughly misleading. The German word "Fibel" corresponds to our "primer," but instead of a slight introduction we have a voluminous tome containing a comprehensive up-to-date discussion of antibiotics. The book is divided into a general section discussing the chemistry and pharmacology of antibiotics (pp. 1–208) and a clinical section arranged by disease entities (pp. 209–785). Good use has been made of tables

and graphs for the effective, condensed presentation of therapeutic indications and results, and for the demonstration of characteristic properties such as tolerance, stability, solubility, dosage forms, etc. The bibliographies at the end of each chapter include all pertinent material published as late as 1953.

Principles and Practice of Antibiotic Therapy.—By HENRY WELCH. Medical Encyclopedia, Inc., New York, 1954. xix + 699 pp. 15 x 22 cm. Price $12.

The author of this comprehensive work is eminently qualified to discuss the field of antibiotics. He has been closely connected with the field from its inception and is now the Chief of the Division of Antibiotics of the U. S. Food and Drug Administration, as well as editor-in-chief of the Journal, *Antibiotics and Chemotherapy*. The book is conveniently divided into two parts, the first of which is concerned with the isolation and development, the antimicrobial activity and the pharmacology of the antibiotics, while the second part is devoted to the clinical uses and sub-divided according to disease entities. Each chapter contains a selected bibliography and there is a satisfactory index. This book can be recommended as the most up to date and complete volume on antibiotics at present available in the English language.

Chemotherapy of Infections. By H. O. J. COLLIER. John Wiley & Sons, Inc., New York, 1954. xvi + 248 pp. Illustrated. 14.5 x 19.5 cm. Price $4.

The chemotherapy of parasitic infections, caused by protozoa, bacteria, and rickettsias, but not by parasitic worms or viruses, and different chemotherapeutic agents are discussed by the author. About one-third of the book is taken up with such topics as microbial attack and body defenses, the problem of getting the chemical to the microbe mechanism faction of antimicrobial substances, and drug resistances. Then penicillin, streptomycin, neomycin, and viomycin are briefly discussed and one chapter more or less routinely deals with the sulfonamides. Tuberculosis, leprosy, some tropical diseases, and the malarias are the only diseases for which the chemotherapy was outlined, these being contained in less than forty pages at the end of the book. *Chemotherapy of Infections* is a rather routine review of the literature and may prove useful for this purpose.

Advances in Enzymology and Related Subjects of Biochemistry. Vol. XV. Edited by F. F. NORD. Interscience Publishers, Inc., New York, 1954. 547 pp. 16.5 x 24 cm. Price $11.

Contributions to Vol. XV are from institutions in England, Germany, Japan, Australia, and France, as well as in this country. The contents include topics on mechanism of various enzyme actions, urea synthesis, the thermodynamics of immunological reactions, the citric acid cycle, the structure of the tobacco mosaic virus, and other subjects. Each contributor seems to cover the subject quite adequately and cites extensive references to publications by many other top persons in the fields, thus providing an almost unlimited source of information on the subject matter included in this volume. The chapters by the contributors from France and Germany are in the French and German languages, respectively.

Statistical Analysis in Chemistry and the Chemical Industry. By CARL A. BENNETT and NORMAN L. FRANKLIN. (Sponsored by The Committee on Applied Mathematical Statistics, The National Research Council.) John Wiley & Sons, Inc., New York (Chapman & Hall, Ltd., London), 1954. xvi + 724 pp. 16 x 23.7 cm. Price $8.

Modern statistical methods in chemistry are being used more extensively during the past few years and it was this increasing interest that prompted the Committee on Applied Mathematical Statistics of the National Research Council to decide that a comprehensive book on applied mathematical statistics with illustrative examples and material from chemistry and the chemical industry should be prepared under the direction of the Committee. The two authors are "a mathematical statistician with some knowledge of chemistry and a chemist with some knowledge of mathematical statistics," and each has had considerable experience in the application of statistical methods to problems in the field of chemistry.

The book deals mainly with the class of statistical methods that involve inferences and the first five chapters are used for developing the basic principles of this approach. In the remainder of the book are found the more specialized methods for the industrial problems that seem to be of greatest importance.

For the understanding of the statistical techniques presented, sufficient mathematical background, not above the level of the engineering or chemistry graduate, is given and examples taken from the industrial field are used to illustrate the application of the methods. Thus this book can be a comprehensible and valuable aid to those in chemistry and allied industries. The authors state that this book could also be used as a textbook for upper class or graduate students in statistics. References to original papers from which examples were selected are included at the end of the book.

Vegetable Tanning Materials. By F. N. HOWES. Butterworths Scientific Publications, Ltd., London, 1953. (American Distributor: The Chronica Botanica Co., Waltham, Mass.) 325 pp. Illustrated. 14.5 x 22 cm. Price $5.50.

This book gives the results of an international survey of some 800 species of plants rich in tannins, the use of which are extended today to deep-oil well drilling, ink and synthetic materials manufacture, boiler-water treatment, less extensively in plastic manufacture, and in medicine, as well as in the leather industry. The sources of the tannins are divided into barks, woods, fruits, leaves, roots, and plant galls. The plants selected were those known to be of use by man somewhere in the tanning of leather and those species with 10 per cent or higher tannin content and which may be potential sources of tannin. Botanical origins of the plants and such topics as cultivation, collection, uses, geographical sources, and other pertinent informa-

tion are discussed under each heading. Considerable attention is given to the mangrove and wattle plants which are considered potential sources of tannin, particularly in view of the diminishing chestnut and quebracho trees.

1954 Medical Progress—A Review of Medical Advances During 1953. MORRIS FISHBEIN, Editor. The Blakiston Co., Inc., New York, 1954. 345 pp. 16.5 x 24 cm. Price $5.

Dr. Fishbein has again assembled a bevy of outstanding specialists in various fields of medicine and, thereby, achieved an authoritative coverage of medical research trends during the last year. Dr. P. D. White is responsible for cardiology, Dr. C. W. Mayo for surgery, Drs. H. A. Rusk and J. G. Benton for rehabilitation, to name just a few. Of particular interest to the pharmacist is the chapter on *Newer Drugs—Their Actions and Uses*, the author of which is Dr. P. L. Werner of the A. M. A. Council on Pharmacy and Chemistry. This paper shows the relatively low number of actually new remedies. It also shows the tendency of current work in the therapeutic field. Says Dr. Werner: "New agents for the control of hypertension, cancer, and kidney diseases make their debut in this annual coming-out party but seem destined for abandonment in the continuing search for curative rather than control measures. Also to be noted is the cyclical revival of older remedies in the form of more purified principles of the crude drugs."

Industrial Fermentations. By LELAND A. UNDERKOFLER and RICHARD J. HICKEY. Vol. I. Chemical Publishing Co., Inc., New York, 1954. x + 565 pp. Illustrated 14.5 x 22.5 cm. Price $12.

These volumes are presented in the form of a symposium with most of the chapters being written by contributors outstanding in either industrial or academic fields. Most topics, with few exceptions, are restricted to industrial processes of commercial importance. The editors state that limitation of processes of commercial importance or potentially important for commercial exploitation was attempted and they appear to have accomplished this aim.

The first part of this volume discusses alcoholic fermentation and its modification, with about one-half of the book being devoted to this topic. Fermentation of grains, molasses, sulfite waste liquor, alcohol from wood waste, the brewing industry, production of wines, and glycerol are discussed. The production of yeast, commercially and for food and feed, is described in the second part while in part three is discussed butanol-acetone fermentations. In the last part about one-fifth of the book contains descriptions of the fermentative production of the organic acids—lactic, citric, gluconic, fumaric, itaconic, and acetic, or vinegar.

The contents of Volume II are given and indicate that this second volume will probably be of as much value as the first volume to workers in the fermentation industry.

BOOKS RECEIVED

Das Chlorophyll in Medizin und Kosmetik. By HANS VOGEL. Verlag Hans Carl, Nuernberg, 1954. 152 pp. 13 x 19.5 cm. Price *DM* 7.50.

Spot Tests. 4th completely revised English edition. By FRITZ FEIGL (translated by Ralph E. Oesper). Vol. I—Inorganic Applications. Vol. II—Organic Applications. Elsevier Publishing Company, New York (London), 1954. Vol. I—518 pp.; Vol. II—436 pp. 16.5 x 23.5 cm. Price, Vol. I—$6.50; Vol. II—$6.25.

The Kinetic Basis of Molecular Biology. By FRANK H. JONSON, HENRY EYRING, and MILTON J. POLISSAR. John Wiley & Sons, Inc., New York (Chapman & Hall, Ltd., London), 1954. 874 pp. 16 x 24 cm. Price $15.

Medical Uses of Cortisone, Including Hydrocortisone and Corticotropine. F. D. W. LUKENS, Editor. The Blakiston Co., Inc., New York, 1954. 534 pp. Illustr. 16 x 23.5 cm. Price $7.50.

1954 Medical Progress—A Review of Medical Advances During 1953. MORRIS FISHBEIN, Editor. The Blakiston Co., Inc., New York, 1954. 345 pp. 16.5 x 24 cm. Price $5

Thérapeutiques Récentes—Antibiotiques, Radio Isotopes, ACTH, Cortisone, Résines Exchangeuses de Cations, Isoniazide Hyperthyroidies. By R. TURPIN, Y. CHABERT, R. FAUVERT, G. BROUET, CL. LAROCHE, H. BRICAIRE, GILBERT DREYFUS, S. BLONDIN, M. ZARA, J.-C. SAVOIE. Masson & Cie, Paris, 1954. 152 pp. 17 x 22.5 cm. *fr.* 800.

Substances Naturelles de Synthèse—Prèparations et Méthodes de Laboratoire. Vol. VIII. LÉON VELLUZ, Editor. Masson & Cie, Paris, 1954. 157 pp. 16 x 22.5 cm *fr.* 2,200

Substances Naturelles de Synthèse—Préparations et Méthodes de Laboratoire. Vol. IX. LÉON VELLUZ, Editor. Masson & Cie, Paris, 1954. 186 pp. 16 x 23.5 cm. *fr.* 2,280.

Springfield City Hospital Formulary. 1st rev. WALTER FRAZIER, Editor. Springfield, Ohio, 1954. 63 pp. 23 x 29 cm. mimeographed, paper bound. No price.

Méthodes et Réactions de l'Analyse Organique. Vol. III. (Réactions Colorées et Fluorescences). LÉON VELLUZ, Editor. Masson & Cie, Paris, 1954. 298 pp. 16.5 x 25 cm. *fr.* 2,750.

Principles and Practice of Antibiotic Therapy. By HENRY WELCH. (Published by Medical Encyclopedia Inc.). Distributed by Blakiston Co., New York, 1954. xix + 699 pp. 15 x 22 cm. Price $12.

Apotekens Register över Standardforpackade Läkemedel, 1954. 17th ed. (Swedish Codex of Materia Medica). Published by Victor Pettersons Bokindustrie Aktiebolag, Stockholm, Sweden, 1954. Under sponsorship of Apotekarsocietetens Laboratorieavdelning Apotekens Kontrollaboratorium (Laboratory Division of the Pharmaceutical Society and Pharmacy Controll Laboratory). 478 pp. + 11 pp. 19 x 23 cm. No price.

Superior Ingredients... For superior products

The AMERCHOLS® are natural, non-ionic OINTMENT BASES AND EMULSIFIERS which contain *Lanolin Cholesterols in their Most Active Form.*

These stable cholesterol and multi-sterol surfactants induce rapid drug release, promote optimum healing rates, and are safe for the most delicate tissues. We know of no case of an allergy due to an AMERCHOL.*

MODULAN™ is a chemically modified LANOLIN EMOLLIENT with unique new properties for use in OINTMENTS AND EMULSIONS. It imparts waxy, protective hydrophobic films; is oil soluble and compatible with O/W emulsions, soaps and shampoos. Clinical investigations indicate that MODULAN IS HYPO-ALLERGENIC.*

**References, technical data and suggested formulations are available from our research laboratories. Please write on your business letterhead.*

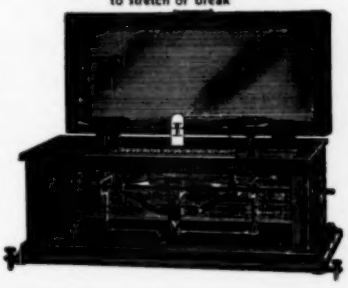
AMERICAN CHOLESTEROL PRODUCTS
INCORPORATED
MILLTOWN NEW JERSEY

Scale illustrated is our
No. 540 all Glass Model

Capacity—4 oz. (120 grams)
Sensitivity—1/10 grain
Finish—Genuine light mahogany with chrome plated parts
Price—$145.00 (subject to change without notice)

PROMPT DELIVERY
on this as well as other class A scales
No. W35 (all glass in lid) $110.00
No. W33 ..... 98.00
All With Stainless Steel Pans

Our scales have no bands or wires to stretch or break



Still the best and lowest priced scale made

HENRY TROEMNER
Manufacturer of the Finest Scales for the Druggist Since 1840
911 Arch Street
Philadelphia 7, Pa.
(Send for Circular A-A)

The Massachusetts College of Pharmacy
Longwood Avenue, Boston

Graduate study in Boston has always appealed strongly to students in the fields of medicine and public health. The environment is stimulating and attracts students from all parts of the world. Library, laboratory, hospital, and other facilities for graduate work are outstanding in this area.

The Massachusetts College of Pharmacy is located in this great medical and educational center. It offers unusual opportunities to graduates of colleges of pharmacy who are interested in further study in pharmacy, pharmaceutical chemistry, and pharmacognosy.

For additional information, write to HOWARD C. NEWTON, Dean

A SPECTRUM IS RECORDED IN 1 MINUTE ... with the Warren Spectracord

The Warren Spectracord brings to spectrophotometry long needed speed-with accuracy in plotting the transmittance of a mixture of compounds. The Spectracord automatically scans and records the entire visible or ultraviolet spectrum in less than a minute . . . and it does it without sacrificing hairline precision in plotting the finest spectral structure.

A curve of average complexity is recorded by the Spectracord in approximately two minutes. The entire operation—inserting the sample, drawing the complete curve, changing the graph paper, inserting a second sample—takes less than two and a half minutes. This enables you to check 170 samples in an eight-hour day with time to spare for preparing samples. (By contrast an analyst is lucky if he can finish two manually-recorded spectra in a day.)

The Spectracord has all the versatility of the tried-and-proved Beckman DU Spectrophotometer, which it uses as a monochromator, plus many features all its own. Merely by flipping a switch, the Spectracord can be converted into a single-beam instrument for studying emission spectra, fluorescence, polarization phenomena and other changes in source-intensity.

SPECIFICATIONS

CAPACITY—The Spectracord operates normally in two ranges: the visible spectrum from 350 to 750 millimicrons, or the ultra-violet range from 215 to 375 mu. It is easily adjusted to cover other ranges, with or without expansion, between 200 and 750 mu.

Preprinted graph paper (11" x 17") which shows each wavelength exactly is available for either the visible or the ultra-violet range.

CONTROL PANEL—The transmittance scale is visible at all times on the control panel and a pointer shows the position of the pen. The wavelength setting and slit adjustment of the monochromator appear through windows.

OPERATION—No batteries are needed; the Spectracord is line-operated from 117 (±10%) volt, 60 cycle a.c. and uses only 250 watts.

Write for FREE brochure FS-245-U which completely describes the Spectracord and its accessories.

FISHER SCIENTIFIC

Boston Chicago Detroit Philadelphia St. Louis Montreal
Buffalo Cleveland New York Pittsburgh Washington Toronto

America's Largest Manufacturer-Distributor of Laboratory Appliances and Reagent Chemicals